Molecular mechanisms of environmental signal reception in mouth-form and dauer polyphenism in *Pristionchus pacificus*

Dissertation

der Mathematisch-Naturwissenschaftlichen Fakultät

der Eberhard Karls Universität Tübingen

zur Erlangung des Grades eines

Doktors der Naturwissenschaften

(Dr. rer. nat.)

vorgelegt von

Maša Lenuzzi

aus Karlovac, Kroatien

Tübingen

2021

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

Tag der mündlichen Qualifikation:	17.12.2021
Dekan:	Prof. Dr. Thilo Stehle
1. Berichterstatter:	Prof. Dr. Ralf J. Sommer
2. Berichterstatter:	Prof. Dr. Oliver Bossdorf

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Summary

Developmental plasticity is the ability of a genome to change its phenotype across heterogeneous environments. Although it is a common phenomenon in nature, the receptive molecular pathways and sensory tissues which integrate environmental signals into development are not well investigated yet. Discrete cases of phenotypic plasticity, commonly known as polyphenisms, are particularly useful for the investigation of molecular regulation of developmentally plastic traits due to the clear relationship between environmental signals and phenotypes as well as due to easy identification of distinct phenotypes. The most comprehensive network of polyphenism regulation is found in dauer polyphenism in the model organism *Caenorhabditis elegans*. Primary cilia of sensory neurons have a major role in the perception of the environmental stimuli which regulate dauer formation in *C. elegans*. However, a comparative approach is necessary to explore general principles. The diplogastrid nematode *Pristionchus pacificus* shares a dauer polyphenism with *C. elegans*, and has an additional polyphenism of mouth-structures (carnivorous and bacteriovorus morph). While the switch network of mouth-form polyphenism is well investigated, the molecular machinery of sensory cilia has not been investigated so far.

This thesis addresses the role of far upstream molecular pathways, which integrate aspects of the environment into development. The first major project included an investigation of the development and role of primary sensory cilia in *P. pacificus* through a candidate approach. Results showed that, while the development of sensory cilia is highly conserved between *C. elegans* and *P. pacificus*, dauers in *P. pacificus* are regulated in a contrasting manner by the receptive molecular pathways expressed in the sensory cilia in *P. pacificus* and *C. elegans*. These results, advance previous findings, which showed the divergence in chemotaxis preferences between the two species" respective dauers and the discovery of the beetle sex pheromone receptor OBI-1 in *P. pacificus*. Our study fleshes out previous speculations that the receptive molecular pathways of polyphenism evolve fast between different species. The discovery of mouth-form phenotype in mutants completely lacking sensory cilia motivated the screen for mouth-form phenotype across previously generated mutants of the components of sensory cilia, which led to discovery of contrasting roles of individual components in mouth-form polyphenism regulation, with anterograde transport presumably having the strongest role in the formation of the carnivorous morph.

The second major project used an unbiased approach via EMS mutagenesis to investigate the receptive pathways which integrate temperature into mouth-form dimorphism which resulted in the discovery of sensory cilia associated-cGMP signalling pathway, a role similar to what has previously been described in the dauer polyphenism in *C. elegans*. Interestingly, as in the case of complete loss of cilia, mutants of this pathway have a contrasting role in dauer formation between *C. elegans* and *P. pacificus*. Both projects showed that different environmental stimuli relevant for determining the mouth-form are integrated far upstream through pleiotropic receptive molecules. This thesis provides a comprehensive comparative description of molecular pathways that regulate mouth-form polyphenism in *P. pacificus*.

Zusammenfassung

Entwicklungsplastizität beschreibt die Fähigkeit eines Genoms, unterschiedliche Phänotypen in unterschiedlichen Umgebungen auszubilden. Obwohl das Phänomen oft in der Natur vorkommt sind die genetischen Mechanismen sowie die Gewebe, die dafür verantwortlich sind, Signal aus der Umwelt in den Entwicklungsprozess zu integrieren, noch nicht genügend erforscht. Diskrete Fälle von Entwicklungsplastizität, auch Polyphänismen genannt, eignen sich aufgrund der klaren Beziehung zwischen Umweltsginal und ausgebildetem Phänotyp sowie der einfachen Identifizierung des Phänotypen besonders gut für die Erforschung der molekularen Steuerung von Entwicklungsplastizität. Das am umfassendsten beschriebene Regulationsnetzwerk eines Polyphänismus unterliegt der Bildung von Dauerlarven im Modellorganismus *Caenorhabditis elegans*. Um die allgemeinen Prinzipien polyphäner Regulation zu verstehen, bedarf es jedoch eines vergleichenden Ansatzes. Auch bei *Pristionchus pacificus*, einer Spezies von Fadenwürmern aus der Familie der Diplogastridae, tritt der Polyphänismus der Dauerlarven auf; zudem verfügt die Spezies über einen weiteren Polyphänismus der Mundform (fleischfressende und bakterienfressende Morphe). Das Netzwerk an genetischen Schaltpunkten dieses Polyphänismus wurde bereits gut untersucht, doch eine detaillierte Beschreibung der molekularen Prozesse an den für Sinnesreize verantwortlichen Zilien blieb bislang noch aus.

In dieser Arbeit untersuche ich die rezeptiven Mechanismen, die der Integration von Signalen aus der Umweltbei der Entwicklung der Polyphänismen in *P. pacificus* zugrundeliegen. Das erste Projekt untersuchte die Entwicklung und Rolle der primären Zilien in *P. pacificus* mithilfe eines Kandidatengen Ansatzes. Obwohl die Entwicklung primärer Zilien in *P. pacificus* und *C. elegans* größtenteils konserviert ist, konnten wir beschreiben, wie *P. pacificus'* Dauerlarven in kontrastierender Weise durch rezeptive molekularen Pfade reguliert werden, die in den sensorischen Zilien ausgedrückt werden.Damit werden frühere Befunde untermauert, die die Divergenz der Chemotaxis-Präferenzen zwischen *C. elegans* und *P. pacificus* Dauerlarven und die Entdeckung des Käfer-Sex-Pheromon-Rezeptors OBI-1 in *P. pacificus* zeigten. Unsere Studie konkretisiert damit frühere Spekulationen über die schnelle Entwicklung molekularer Signalwege von Polyphänismen zwischen verschiedenen Arten. Die Entdeckung des Mundform-Phänotyps bei Mutanten, denen die sensorischen Flimmerhärchen völlig fehlen, motivierte das Screening auf den Mundform-Phänotyp bei zuvor erzeugten Mutanten der Komponenten der sensorischen Flimmerhärchen. Dies führte zur Entdeckung unterschiedlicher Rollen einzelner Komponenten bei der Regulierung des Mundform-Polyphänismus, wobei der anterograde Transport vermutlich die Hauptrolle bei der Bildung des fleischfressenden Morphs spielt.

Das zweite große Projekt verwendete eine EMS-Mutagenese, um die rezeptiven Wege zu untersuchen, die Umgebungstemperatur in die Ausbildung des Mundform-Dimorphismus integrieren, was zur Entdeckung des sensorischen Zilien-assoziierten-cGMP-Signalweges führte, dessen Rolle zuvor in *C. elegans'* Dauerlarven beschrieben wurde. Interessanterweise weisen Mutanten dieses Signalweges, wie auch im Fall des vollständigen Verlustes der Zilien, eine gegensätzliche Rolle bei der Dauerpolyphenese in *C. elegans* und *P. pacificus* auf. Beide Projekte zeigen, dass verschiedene Umweltreize, die die Mundform beeinflussen, weit upstream durch pleiotrope rezeptive Moleküle integriert werden. Diese Arbeit liefert eine umfassende vergleichende Beschreibung der molekularen Signalwege oberhalb der genetischen Schalter, die den Mundform-Polyphenismus bei *P. pacificus* regulieren.

Introduction

1.1 Developmental plasticity

The ability of the genotype to respond to its environment by modifying its development is called developmental (phenotypic) plasticity. Developmental plasticity is ubiquitous in nature (West-Eberhardt, 2003). However, the genetic mechanisms of integration of environmental signals into the development are not well investigated.

Plastic responses can be described and investigated using a reaction norm (*Reaktionsnorm*) - a function of phenotypes change across different environments. Reaction norms provide an extremely useful tool for the comparative research of developmental plasticity, allowing us to visualize and compare plastic responses between individuals and populations (Woltereck, 1909; Bradshaw, 1965; Pigliucci, 2001). While continuous plasticities are more common in nature, discrete cases, also called polyphenisms, are more amenable to research, due to a binary output and clearer distinction between genetic and environmental components (Shapiro, 1976; Simpson et al., 2011; Sommer, 2017; Yang and Pospisilik, 2019; Sommer, 2020).

Plastic responses can be induced by both biotic factors, such as population density or predation, and abiotic factors, such as temperature or humidity. For example, increased population density promotes melanization and migratory behavior in locusts, which allows them to migrate to other areas and find new food sources, while temperature and day length together act in regulation of seasonal polyphenism in butterflies (Nijhout, 1999; Pener and Simpson, 2009). Signals from the environment can be perceived either specifically, e.g. through specific receptors on the surface of the animal, or can produce global changes in e.g. metabolic rate or growth speed, to alter the phenotype (Nijhout, 1999). While predator-induced spine formation in rotifers is induced by the presence of specific predator pheromones or kairomones in the water, rich larval diet in dung beetles alters the global metabolic changes which leads to a larger size of the beetle during the last molt and facultative horn expression (Gilbert, 1999; Moczek and Emlen, 1999).

In their book "Ecological developmental biology", Gilbert and Epel (2015) provide a profound overview on the latest state of the art research of molecular mechanisms of

developmental plasticity. They differentiate between three ways in which environmental signals are integrated into the development to change the phenotype:

1. Direct transcriptional regulation of genes: the regulation of opening and closing of the chromatin by DNA and histone modifying enzymes, which leads to expression or repression of target genes.

2. The neuroendocrine system: sensory neurons or synaptic transmission by activating secretory glands, which secrete hormones that bind to their receptors, resulting in translocation of the receptor from the cytoplasm into the nucleus where it binds the promoters of target genes

3. Direct induction: cell-to-cell communication triggers the signalling cascade, which leads to expression of target genes, e.g. antigen-induced B-cell differentiation between plasma and memory cells (Figure 1).

There has been an increase in the prominence of research on phenotypic plasticity over the last few decades (Kelly et al., 2012). This arises as a consequence of 1) development of methodologies such as molecular cloning and quantitative genetic methods, which enabled scientists to investigate the genetic mechanisms behind the reaction norms and 2) the changes in the environment caused by the dawn of the Anthropocene serve as a great motivation for biologists to study the ways in which organisms can accommodate themselves to a rapidly changing environment (Morange, 2009; Refsnider and Janzen, 2016; Bonamour, 2019; Catullo et al., 2019; Richard et al., 2019). One of the major challenges is a global increase in environmental temperature (Hougton, 2001; Root et al., 2005; Vitousek, 1994; Parmesan and Yohe, 2003; Rosenzweig et al., 2008).

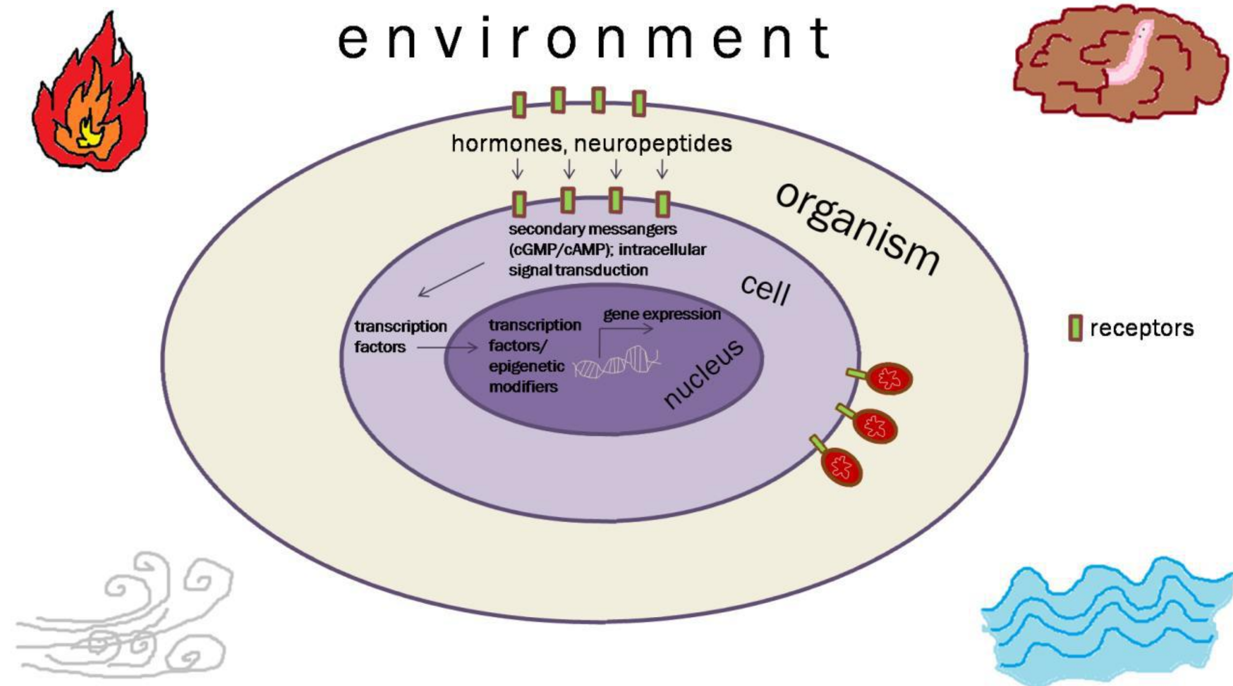


Figure 1: Schematic representation of the molecular mechanisms of integration of environmental signals according to Gilbert and Epel, 2015. Environment is perceived through receptors of sensory cells (Song et al., 2020; Marshall et al. 2016; Baldwin et al., 2020; Bargmann, 2006). Hormones and neuropeptides mediate intercellular communication and transfer signals into the target tissues by binding to the receptors on their surface (Beato, 1993; Norman and Litwack, 1997; Nijhout, 1998; Strand, 1999). Receptor activation triggers various intracellular signalling cascades leading to activation of various types of transcription factors which regulate gene expression in the nucleus. Epigenetic modifiers, such as histone and DNA modifying enzymes and/or small RNAs and Argonaute proteins, also regulate gene transcription in the nucleus through chromatin modifications (Whiteside and Goodbourn, 1993; Biel et al., 2005; Weigel and Moore, 2007; Li, 2014; Lambert et al., 2018). Bacterial cells (dark red) are directly inducing host cells.

1.1.1 Mechanisms of temperature perception by polyphenic species

Temperature is one of the major abiotic factors that shape development (Hochachka and Somero, 2002; Sunday et al., 2010). There are several examples of temperature-dependent polyphenism, among which the best description of molecular pathways is found in temperature dependent sex determination in reptiles, seasonal dimorphism in butterflies and dauer formation in the model nematode *Caenorhabditis elegans* (reviewed in Fielenbach and Antebi, 2008; Nylin, 2013; Martínez-Juárez and Moreno-Mendoza, 2019). Polyphenic animals are among the most vulnerable to global warming because they often depend on a narrow temperature range at a specific time of the year in order to produce well-adapted adult

morphologies (Oostra et al., 2018; Janzen, 1994). The investigation of the signaling pathways responsible for integrating environmental temperature is crucial for our understanding and anticipation of organisms" coping with future changes in global temperatures brought by climate change (Refsnider and Janzen, 2016; Bonamour, 2019).

Temperature-dependent sex determination (TSD) is common among reptiles. Two types of reaction norms, Pattern I and II, are found in TSD (Jenzen and Krenz, 2004). In Pattern I, one sex is produced at low and the other sex at high temperatures. In red-eared slider turtles, females are produced at lower temperatures than males (Wibbels et al., 1998). In Pattern II, females are produced at extreme temperatures. In crocodiles, extreme temperatures produce females while more moderate ones produce males (Lang and Andrews, 1994). Temperature affects levels of aromatase (*cyp19a1*), an enzyme, which converts testosterone to estradiol, thereby controlling the ratio of oestrogen and testosterone in the developing gonads of the embryo. In some turtles, aromatase inhibitors turn all embryos into males (Desvages et al., 1993; Dorizzi et al., 1994; Pieau et al., 1994). In the red-eared slider turtle *Trachemys scripta*, DNA methylation is progressively removed from the aromatase promoter during female development, indicating active regulation by upstream epigenetic enzymes. However, the nature of these enzymes remains largely elusive (Matsumoto et al., 2016). Recently, Ge et al., 2017, 2018) found that estrogen signaling leads to repression of the transcription factor Dmrt2 and its positive regulator histone demethylase KDM6B, specifying female sex in *T. scripta*. In the Australian central bearded dragon (*Pogona vitticeps*), exposure to high temperature overwrites the genetically determined sex and all embryos develop into fully functional females,which is regulated by an alternative transcript of the JARID2 component of the chromatin regulator Polycomb Repressive Complex 2. Interestingly, the mammalian sex-deterimining gene SRY is regulated by a related type of molecule (Deveson et al., 2017). TRPV4 receptor, a TSD regulator of male development in *Alligator mississippiensis*, is the only factor upstream of hormone signaling identified to our knowledge (Yatsu et al., 2015).

Among insects, temperature was co-opted as a signal in seasonal polyphenism in butterflies and in regulation of coloration in the dispersal polyphenism in locusts (Brakefield and Reitsma, 1991; Nijhout, 1999; Dong et al., 2013; Tanaka et al., 2012). The wet season form of the butterfly *Bicyclus anynana* has brighter wings with clearly visible eye-spot patterns on the wing edges, while the dry season form has darker wings with less visible eye-spots (Brakefield and Reitsma, 1991). The presence of eye-spots is regulated by a conserved homeodomain transcription factor *Distal-less* (Dll). While both larvae grown on 20°C and 24°C had similar

expression patterns of *Distal-less* in the early development, the patterns diverged at the later stages, and this change was attributed to different levels of the molting hormone 20-hydroxyecdysone (20E). High temperatures increase the amount of 20E, which leads to stabilization of the Dll expression in the target tissues. At low temperatures, 20E is not produced and Dll expression fades in the later larval stages, inducing dry-season morphology (Carroll et al., 1994; Beldade et al., 2002). Dll activates Notch, a highly conserved membrane receptor involved in tissue patterning in animals (Reed and Serfas, 2004). Comparative studies indicated conservation of the pathway among different genera (Koch and Bückmann, 1987; Dhungel et al., 2016; Reed and Serfas, 2004). In the locust *Schistocerca gregaria*, yellow and black body coloration is associated with dispersal morph, while solitary form displays various body coloration. Temperature regulates continuous plasticity in gregarious morph. Neuropeptide corazonin (Crz) and high temperature antagonistically regulate yellow coloration of gregarious morph through regulation of yellow protein of the takeout family (YPT) (Sugahara and Tanaka, 2018). Besides all these examples, the genetic mechanisms of temperature integration in developmental switching are indisputably the best studied in the case of dauer polyphenism in *C. elegans*, which will be described below.

1.2 Dauer polyphenism in the model nematode *C. elegans*

C. elegans, like the other nematodes, undergoes four molts before reaching the adult stage (Bird and Bird, 2012). If it encounters harsh or stressful environments, *C. elegans* can switch its development to form an alternative, dauer stage after the second molt. Dauer larvae are a specialized alternative juvenile stage, common among rhabditid and diplogastrid nematodes, which evolved as an adaptation to survival in adverse environmental conditions and/or dispersal *via* hosts (Sudhaus and von Lieven, 2003; Warburton and Zelmer, 2010). Dauers were first described by the German researcher Anton G. Fuchs (1937), who isolated them from bark beetles and coined the name *Dauer-Larven*, meaning continuous or enduring larvae. *C. elegans* dauers have specialized morphological and physiological features, such as a tick cuticle and a feeding preventing mouth-plug, and their metabolism depends mostly on glycolysis and fermentation as opposed to later larval and adult stages, which are mostly fueled by aerobic respiration (Cassada and Russell, 1975; Riddle et al., 1981; Wadsworth and Riddle, 1981; Holt and Riddle, 2003). Because of its ability to survive stressful environmental conditions and its strikingly long life span, dauer larva formation has been established as a model system for research on the molecular mechanisms of resistance and longevity (Kenyon, 2011). This

research has led to a rich literature on the genetics of dauer and one of the best descriptions of genetic regulation of polyphenism (Fielenbach and Antebi, 2008). Unfortunately, however, dauer research has not focused on investigating polyphenism from an evolutionary and comparative perspective within the broader phenomenon of phenotypic plasticity.

Environmental stimuli controlling dauer entry are perceived by sensory neurons (reviewed in Hu, 2007). *C. elegans* has 60 ciliated sensory neurons, many of which have ciliary endings (Inglis et al., 2007). These primary cilia are highly conserved organelles among eukaryotes and are involved in the sensing of chemical and physical signals from the extracellular space. They are composed of nine microtubule doublets that extend from a centriole and bind several proteins, namely intraflagellar transport (IFT) proteins, which are involved in receptor trafficking and structural maintenance (Rosenbaum and Witman, 2002; Pazour, et al., 2005; Satir et al., 2010). Primary cilia perceive the signals involved in dauer regulation and mutants of ciliary components display contrasting effects on dauer polyphenism, indicating both promoting and inhibitory roles of cilia in dauer regulation (Perkins et al., 1986; Vowels and Thomas, 1994; Jenzen et al., 2010). The formation of cilia is dependent on the single transcriptional factor *daf-19/*RFX, which directly regulates expression of IFT genes. In *daf-19* mutants, sensory neurons completely lack functional cilia and display many sensory defects (Perkins et al., 1986; Malone and Thomas, 1994; Swoboda, 2000; Senti and Swoboda, 2008). Interestingly, *daf-19* mutants constitutively form dauer even under non-stimulative conditions, suggesting that the cumulative effect of the cilia inhibits dauer formation (Perkins et al., 1986; Malone and Thomas, 1994; Senti and Swoboda, 2008).

The genetic mechanisms of dauer regulation were discovered following mutagenic screens for dauer constitutive (Daf-c) mutants (such as *daf-19*), which produce dauer even in the non-stimulative conditions, and dauer defective (Daf-d) mutants, which have reduced dauer formation even in dauer-promoting environments. Dauer-regulating signals from the sensory cilia are integrated through guanylyl cyclase signaling and three highly conserved endocrine pathways: insulin-like, Transforming Growth Factor (TGF)-ß and steroid hormone signaling (reviewed in Hu, 2007).

Membrane-bound guanylyl cyclases are conserved sensory transmembrane proteins, which contain an extracellular ligand-binding domain, a transmembrane domain and intracellular domains. The latter have guanylyl cyclase catalytic activity, converting intracellular GTP to cGMP. cGMP acts as a second messenger and activates different downstream

signaling molecules, such as protein kinases or cGMP-gated ion channels (Bargmann, 2006; Davies, 2006; Kleppisch and Feil, 2009; Inser and Maathius, 2018).

Insulin signaling is involved in many processes, such as development, longevity, stress resistance, behavior and metabolism (Kenyon, 2011; Rains and Jain, 2011; Snell-Rood and Moczek, 2012; Zhao and Campos, 2012). The main insulin-like growth factor receptor (IGF1R) *daf-2* suppresses dauer formation and promotes growth in favorable conditions (Kimura et al., 1997). Also, several other genes in this pathway have been described to play a role in dauer polyphenism, such as *age-1* and *akt-1* (Malone et al., 1996; Ailion and Thomas, 2003).

The TGF-ß pathway is an ancient pathway involved in the regulation of various biological processes, such as development, growth, homeostasis and immunity (Newfeld et al., 1999; Massagué et al., 2000; Moses et al., 2016). As indicated by the fact that mutants in these genes have the Daf-c phenotype, the *daf-7* TGF-ß ligand, as well as TGF-ß receptors *daf-1* and *daf-4*, suppress the dauer phenotype (Georgi et al., 1990; Estevez et al., 1993; Ren et al., 1996; Schackwitz et al., 1996).

Further downstream, dauer larvae are regulated by a steroid hormone pathway (Hu, 2007). Steroid hormone ligands including several forms of dafachronic acid negatively regulate dauer induction by binding and inhibiting the nuclear hormone receptor *daf-12/*NHR, a vitamin-D receptor homolog, in the cytoplasm (Antebi et al., 2000). Those ligands are synthesised from cholesterol by a cytochrome P450 enzyme, encoded by the gene *daf-9* (Jia et al., 2002; Motola et al., 2006). Additionally, cholesterol deprivation promotes *C. elegans* entering dauer (Gerisch et al., 2001). Integration of insulin-like signalling and steroid hormone signaling occurs in epidermal XXX cells, which resemble neuronal cells, and the hypodermis, both of which express *daf-9* and *daf-2* (Okhura et al., 2003; Gerisch and Antebi, 2004). Following activation, DAF-12 acts as a transcription factor, inducing target gene expression to promote growth and maturation (Antebi, 2000; Aranda and Pascual, 2001).

Another transcription factor downstream of insulin signaling, DAF-16/FOXO, promotes dauer formation (Carter and Brunet, 2007; Kenyon, 2011). DAF-16 physically interacts with the chromatin remodeling complex SWI/SNF in the nucleus to recruit it to chromatin, leading to chromatin remodeling and expression of dauer inducing pathways (Riedel et al., 2013). Dauer larvae have an altered chromatin state compared to other stages and differentially express some genes involved in chromatin remodeling, such as H1 histones. The putative telomere-associated protein *tts-1* is highly overexpressed in dauers compared to non-dauers (Jones et

al., 2001). Hall et al. (2013) observed reduction of two euchromatic marks, H4panAc and H3K4me3, over multiple loci, showing that dauers have a unique chromatin state. Reduction in function of the argonaute protein CSR-1 rescues the deposition of these marks over some of the loci, indicating the role of 22G-RNA class small RNAs (Claycomb et al., 2009; Hall et al., 2013). Transition through dauer stage alters the chromatin state of the post-dauer animals, showing that post-dauers retain the memory of their previous phase (Hall et al., 2010).

C. elegans senses complex environments and integrates different cues into the dauer switch (Golden and Riddle, 1984a; 1984b). For example, the relative amounts of food and pheromones determine dauer induction (Golden and Riddle, 1984a). Higher temperatures lead to increases in the production of and sensitivity to pheromones, but can also induce dauer in an independent manner (Golden and Riddle, 1984b). Temperature and pheromones are perceived by sensory neurons (Bargman, 2006; Mori and Ohshima, 1995). *C. elegans* dauer pheromone, also called daumone, consists of a complex mixture of small lipophilic molecules called ascarosides (Jeong et al., 2005; Butcher et al., 2007). Daumone synthesis is dependent on *daf-22*, and mutations in this gene abolish pheromone production (Golden and Riddle, 1985; Butcher et al., 2009). Dauer-regulating pathways obtained from Daf-c and Daf-d mutants provided the great body of literature on polyphenism regulation. However, the significance of particular pathways in the context of specific environmental signals is not yet clarified. Because of the sensitivity to the different levels of specific ascarosides and the epistatic interactions between different compounds, pathways of integration of daumone into dauer switches have been complicated to study (Hu, 2007). Ambient temperature can be most easily regulated. This led to the thorough mechanistic description of high temperature-induced dauer formation.

1.2.1 Genetic regulation of High temperature induced dauer formation (Hid) in *C. elegans*

Temperature affects many aspects of the behavior and physiology in *C. elegans*. Growth rate, locomotion and reproductive success are all known to be affected by temperature (Klass, 1977; Martin et al., 2013; Zhang et al., 2015). *C. elegans* enters dauer more readily toward higher temperatures within growth range and a slight increase in dauers formed below 15°C indicates a convex reaction norm (Golden and Riddle, 1984a; 1984b). Temperature increases the sensitivity of the nematodes to pheromones, but it also acts in the pheromone-independent way (Golden and Riddle, 1984b; Ailion and Thomas, 2003). The laboratory strain

N2 and natural variants of *C. elegans* are sterile at temperatures above 25°C (Matsuba et al., 2013, Hirsh et al., 1976, Anderson et al., 2011, Petrella, 2014).

The low induction of dauer is visible at 25°C, while at 27°C, wild-type N2 is more clearly Daf-c (Golden and Riddle, 1984b; Ailion and Thomas, 2000). This shift following a 2°C increase in the temperature is called *H*igh temperature *i*nduction of *d*auer (Hid) phenotype. Interestingly, phenotyping at 27°C revealed numerous novel genes at which some mutants formed dauer constitutively, but did not display dauer phenotype at lower temperatures or were defective for dauer formation (Ailion and Thomas, 2000; 2003).

In *C. elegans*, three types of sensory neurons, namely the thermosensory and CO_2 sensory AFD type, the odorsensory, photosensory, electrosensory and thermosensory AWC type and the chemosensory and thermosensory ASI type, are required for a proper response to temperature gradients (Altun and Hall, 2015). Ablations of individual neurons revealed the role of AFD and ASI in dauer induction at 20°C, yet only ASI seems to be necessary for dauer induction on high temperatures (Bargmann and Horvitz, 1991; Ailion and Thomas, 2000). ASI neurons express the guanylyl cyclase *daf-11* (Birnby et al., 2000). *daf-11* mutants form 100% dauers at temperatures above 25°C (Ailion and Thomas, 2000). *daf-11* is involved in the sensing of several environmental signals, including temperature, and is expressed in the cilia of five sensory neurons: ASI, ASJ, AWC, AWB and ASK (Vowels and Thomas, 1994; Birnby et al., 2000; Beckert et al., 2013). The *daf-11* Daf-c phenotype is rescued by ablation of ASJ neuron and to a lesser extent ASE neuron on 25°C, while ASK, ASE and AFD neuron ablations result in moderate rescue (Schackwitz et al., 1996). *daf-25*, an ortholog of mammalian Ankmy2, is required for proper localization of *daf-11* to the sensory cilia and mutants replicate the *daf-11* mutant phenotypes (Jensen et al., 2010). Temperature sensing in AFD, AWC and ASI is dependent on cGMP-dependent cation channel TAX-2/TAX-4. TAX-2/TAX-4 activation by temperature change leading to increasing Ca2+ levels in neurons (Ramot, 2008). The *tax-2* and *tax-4* mutants show weak dauer constitutive phenotype on high temperatures and they suppress strong dauer constitutive phenotypes of *daf-11* (Coburn et al., 1998). Since neither *tax-2* nor *tax* mutants fully replicate the *daf-11* Daf-c phenotype, there are additional targets of *daf-11* cGMP signalling (Coburn et al., 1998; Ailion and Thomas, 2000). The DAF-11/TAX-4/TAX-2 pathway activates insulin and TGF-ß signalling (Murakami et al., 2001; Li et al., 2003).

Insulin-like and TGF-ß pathways suppress dauer formation at high temperatures. Dauer constitutive phenotypes at 27°C are surprisingly rich for mutants in the insulin signaling

pathway, revealing the role of *pdk-1* and *akt-1* downstream of *daf-2* and *age-1* mutants, as well as the role of *aex-6*, *hid-1*, and *hid-2* in dauer regulation on high temperatures. Dauer constitutive phenotype of insulin mutants can be rescued by removing the *daf-16* transcription factor (Ailion and Thomas, 2003). The level of ASI expressed dauer-repressing TGF-ß molecule *daf-7* is repressed at high temperatures (Schackwitz et al., 1996). *daf-11* positively regulates expression of *daf-7* in ASI neurons at 25°C, presumably through inhibition of structural components of cilia *che-3* and *che-2* (Murakami et al., 2001). *daf-3*/SMAD mutants, which is the negative regulator of TGF TGF-ß pathway, are defective for dauer production at 25°C, but they form constitutive dauer at 27°C (Ailion and Thomas, 2003).

The deep insight into the dauer larvae regulation in *C. elegans* we have today has not yet been replicated in any other polyphenism. However, comparative research is necessary in order to investigate the generalities of the mechanisms. The diplogastrid nematode *Pristionchus pacificus* has been established as a comparative model system to *C. elegans* in evolutionary developmental biology (Sommer, 2015). In addition to the dauer polyphenism, a unique mouth-form polyphenism is found in *P. pacificus*, which enables the comparison of the molecular mechanisms of polyphenism, as well as their evolution (Ogawa et al., 2009; Bento et al., 2010).

1.3 *P. pacificus* as a model system for polyphenism

P. pacificus is a cosmopolite species commonly found in nature in necromenic association with scarab beetles and, occasionally, free-living in the soil (Herrmann et al., 2007; Meyer et al., 2017). It is a self-fertilizing hermaphrodite, easily cultured in the lab on *E. coli*, with a short generation time of only ~4 days, and a large brood size, which made it a promising model organism for genetic research (Sommer et al., 2000). The genome of the reference strain PS312, which is ~158Mb in size, has been sequenced in-depth and made available in an online browser (Dietrich et al., 2008; Rödelsperger et al., 2017). The ability to freeze the nematodes enabled a large collection of hundreds of natural variants of which 104 had their whole genome sequenced (Rödelsperger et al., 2014; Pires-daSilva, 2018).

The relative easiness of experimental manipulation of *P. pacificus* led to an establishment of a vast range of molecular and genetics tools, including molecular cloning and CRISPR-Cas9.

1.3.1 Dauer polyphenism in *P. pacificus*

P. pacificus has been established as a comparative model to the dauer polyphenism in *C. elegans* (Ogawa and Brown, 2015). As *C. elegans*, *P. pacificus* can form the facultative dauer larva after the second molt (Figure 2). Unlike *C. elegans* dauer, which are usually found in the soil and occasionally in association with isopods and molluscs, *Pristionchus* dauers are commonly found on scarab beetles (Herrmann et al., 2007). *C. elegans* probably has a phoretic association with their hosts (Félix and Duveau, 2012). In contrast, *P. pacificus* probably has a necromenic association, where it resides on a host beetle in the dauer stage until the host dies and feeds on the bacteria and other nematodes proliferating on the cadaver. *P. pacificus* dauers continue development 4 to 7 days after the death of the beetle. The first re-entry to the dauer can be found on the carcass after 11 days (Meyer et al., 2017).

C. elegans and *P. pacificus* have different chemosensory attraction profiles.While *P. pacificus,* although there is a strain-specific variation in sensitivities, is attracted to plant volatiles, insect pheromones and cuticular hydrocarbons, *C. elegans* is often repelled by plant and insect smells (Hong and Sommer, 2006; Hong et al., 2008). Interestingly, the beetle sex pheromone ZTDO, which is toxic for the developing embryos and larvae and inhibits exit from the dauer stage, attracts adult nematodes. The lipid-binding domain containing protein OBI-1 is regulating the beetle sex pheromone perception in *P. pacificus* (Cinkornpumin et al., 2014).

Different strains of *P. pacificus* show different sensitivities to pheromones of their own and other species. Dauer induction seems to be a competitive strategy between different strains (Mayer et al., 2011; Bose et al., 2014). Dauer sensitivity among *P. pacificus* strains is regulated by the orphan gene *dauerless*, which suppresses dauer formation (Mayer et al., 2015). The lab derivative of the Californian wild-type strain RS2333 has two copies of *dau-1*, and a reduced sensitivity to dauer pheromone. In contrast, the Ohio strain RS5134 has only a single copy of *dau-1* and forms dauers more easily. Interestingly, this gene is expressed in CAN neurons, which are essential in *C. elegans*. Ablation of CAN neurons *P. pacificus* is not lethal but leads to dauer constitutive phenotype (Mayer et al., 2015). Intraspecific pheromone interactions in *C. elegans* remain to be investigated.

The first comparative genetic studies on dauer regulation of *P. pacificus* confirmed the conserved role of the nuclear hormone receptor *daf-12* and steroid hormone dafachronic acid in the regulation of dauer formation (Ogawa et al., 2009). *daf-12* mutants are Daf-d in *P. pacificus* as in *C. elegans* and the administration of the steroid hormone dafachronic acid Δ7-DA could

suppress dauer formation of *P. pacificus* Daf-c mutants (Ogawa et al., 2009). The transcriptional factor *daf-16* has a partially conserved function in *P. pacificus*. In both species of nematodes, *daf-16* is enhancing the formation of dauer and is also involved in formation of morphological characters. However, in *P. pacificus*, it is required for proper remodelling of the pharynx in dauers (Ogawa et al., 2011), while in *C. elegans* neuronal and vulval tissue defects have been observed, indicating a shift in targeted morphologies (Christensen et al., 2011; Karp and Grenwald, 2013).

The *daf-22* gene in *C. elegans* is essential for the production of dauer pheromone (Golden and Riddle, 1985; Butcher et al., 2009). In *P. pacificus*, the *daf-22* gene is duplicated and one duplicate diverged to induce expression of a subset of pheromones under starvation (Markov et al., 2016). Comparative transcriptome analysis between *P. pacificus* and *C. elegans* dauer *vs.* dauer-exit expression patterns revealed both overlap, but also substantial divergence in gene expression patterns between the two species. Neuropeptide-signaling molecules had the most conserved expressions, and most divergent were genes involved in molting cycle and cuticle formation (Sinha et al., 2012).

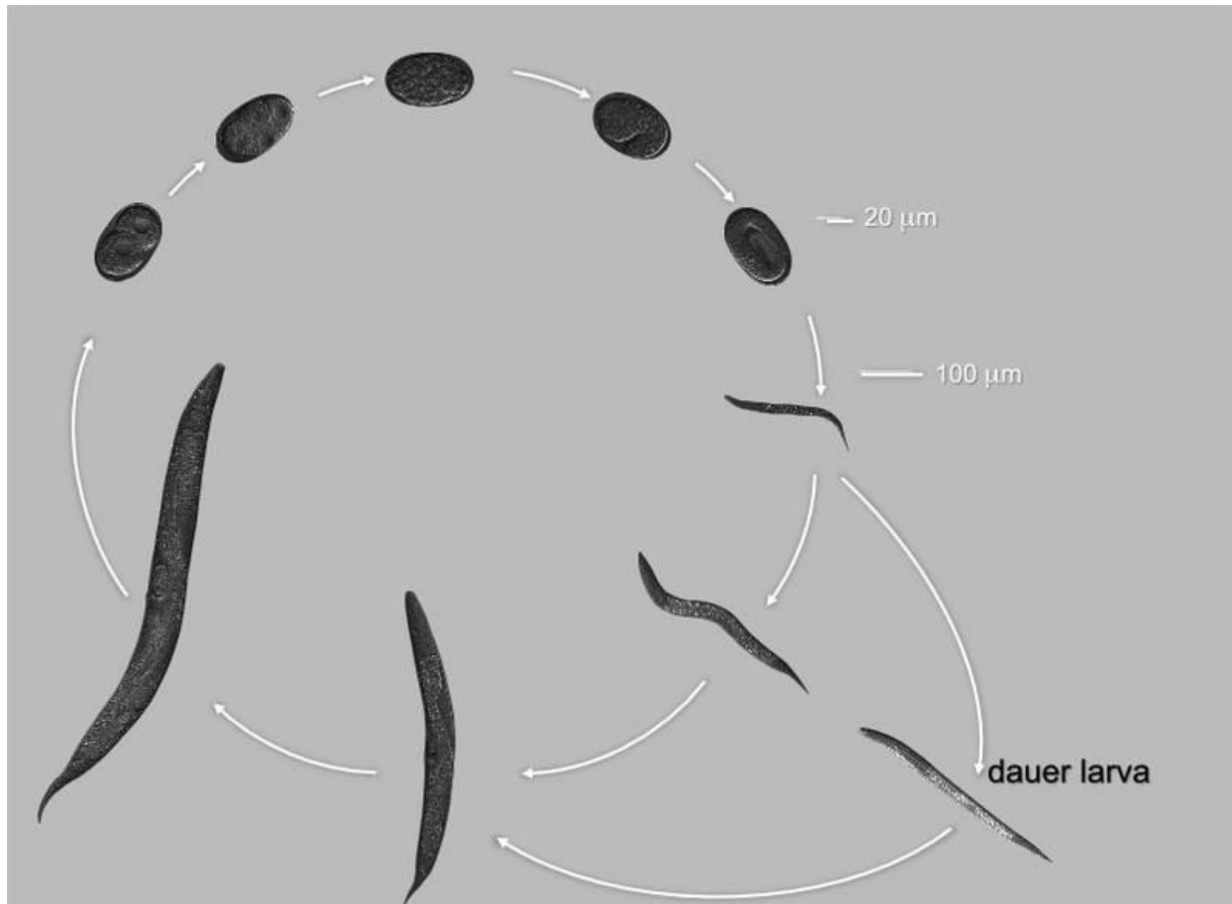


Figure 2: Alternative life cycles in diplogastid nematode *Pristionchus pacificus*. In favourable environments, *P. pacificus* undertakes direct development into adulthood (approx. 4 days) (Sommer et al., 2000). In adverse environmental conditions, such as crowding/starvation, *P. pacificus* takes alternative dauer development. Dauer larvae have a thick protective cuticle and mouth-plug which prevents feeding, as well as slow metabolism. *P. pacificus* dauers are dispersed by scarab beetles (Ogawa and Brown, 2015). © Ralf Sommer/MPI for Developmental Biology

1.3.2 Mouth-form polyphenism in *P. pacificus*

Besides dauer polyphenism, *P. pacificus* also has two distinct feeding morphotypes (Sommer et al., 1996). The eurystomatous (Eu) morph has a wide mouth with two movable, sharp teeth. The other, stenostomatous (St), morph has a deeper and narrower mouth with a single, flapped tooth (Figure 3) (Bento et al., 2010). Both morphs can feed on bacteria, but only the Eu form can also predate on other nematodes. When only bacterial food is available, the St morph grows slightly faster than the Eu morph, with this difference in speed being most pronounced during the last molt, when morph-specific mouth structures are formed (Serobyan

et al., 2013; Wilecki et al., 2015). In recent years, the mouth-form dimorphism in *P. pacificus* has been established as a model for the investigation of polyphenism (Ragsdale, 2015; Yang and Pospisilik, 2019). A little bit over one decade of research led to discovery of numerous genetic and epigenetic regulators, as well as evolutionary aspects of polyphenism and its ecology.

The mouth-dimorphism in *P. pacificus* developed from an ancestral condition-dependent mouth-form dimorphism observed in more basal diplogastrid lineages (Sudhaus and von Lieven, 2003; Susoy et al., 2015). However, it appears that the mouth-form in *P. pacificus* is not only condition dependent, but also stochastic for in standard laboratory conditions both morphs are present in a more or less constant ratio (Bento et al., 2010; Susoy et al., 2016). Most strains show a high Eu:St ratio, but several strains are ~50:50 or highly St (Ragsdale et al., 2013). The presence of both forms facilitated the investigation of theoretically predicted switch genes, which translate continuous environmental information into discrete alternative phenotypes (Bradshaw, 1965; Stearns, 1989; West-Eberhardt, 2003).

The first gene found to play a role in this mouth-form dimorphism is the one-to-one orthologue of *C. elegans' daf-12* gene, a transcription factor which binds delta 7-DA and is involved in dauer polyphenism. *Ppa-daf-12* mutants had more St animals than the wild-type, while treatment with delta 7-DA increased the number of St worms in wild-type animals (Figure 3). Together these two findings indicate that *Ppa-daf-12* is inducing Eu expression (Bento et al., 2010). Ragsdale et al. (2013) conducted a first forward genetic screen to isolate highly St or Eu mutant lines in the laboratory reference strain PS312. This led to identification of the first master switch molecule *eud-1*, located on the X chromosome. *eud-1* mutants completely fail to produce the Eu morph, while transgenic animals carrying a *eud-1* rescue construct fully recover wild-type phenotype (Figure 3). *eud-1* is a homologue of the *C. elegans* sulfatase 2 (*sul-2*) gene. Recently, it was found that *sul-2* negatively regulates sulfated steroid hormones and increases longevity. Sulfated steroid hormones act downstream on *daf-12,* imitating the increase of lifespan after gonad depletion (Pérez-Jiménez et al., 2019). Complementation experiments showed that *eud-1* acts in a dosage-dependent manner. PS312 hermaphrodites have two X chromosomes and are highly Eu, however, males, which have only one X chromosome, are highly St (>70%). Haploinsufficiency of *eud-1* possibly contributes to this difference. *eud-1* is expressed in several sensory neurons or interneurons, whose identity is not yet known (Ragsdale et al., 2013; Kieninger et al., 2016; Serobyan et al., 2016; Sieriebriennikov et al., 2018).

Two epigenetic players upstream of *eud-1*: methyl-binding-protein *mdb-1* and histone acetyltransferase *lsy-12* (Serobyan et al., 2016). *Ppa-mbd-1* and *Ppa-lsy-12* mutants are strongly deficient in the Eu form, possibly because they harbour defects in histone acetylation at the *eud-1* locus and possibly other Eu inducing loci(Figure 3). Indeed, *eud-1* expression is reduced in *mbd-1* and *lsy-12* mutants. *Ppa-lsy-12* also regulates an antisense lnc-RNA transcribed from the opposite strand of *eud-1*. This *as-eud-1* element enhances the expression of *eud-1* (Figure 3).

Suppressor screens in a *eud-1* mutant background resulted in the isolation of three mutants, which reversed the St phenotype of *eud-1* mutant to 100% Eu. The mapping of the causative genetic mark resulted in the identification of three non-synonymous mutations in the nuclear hormone receptor *nhr-40* (Kieninger et al., 2016). However, a recent CRISPR knock-out of *nhr-40* surprisingly had 100% St phenotype, indicating that the original EMS mutants are gain of function alleles and showing that *nhr-40* is a master switch necessary for Eu morph induction (Sieriebriennikov et al., 2020). Kieninger et al. (2016) found that *nhr-40* is expressed broadly, in several somatic and pharyngeal neurons, vulva and the tail using fluorescence in situ hybridization (FISH), while a 2kb promoter construct used by Sieriebriennikov et al. (2020) showed expression in the head, in the putative arcade cell and in hypodermal cells. Both studies reported no overlap in the localization of *eud-1* and *nhr-40*.

In addition to master switches, several other modifiers and regulators of mouth-form polyphenism have been identified. The role of the sulfotransferase *sult-1* (sulfotransferase-1) /*seud-1* (suppressor-of-eud-1) in the mouth-form switch was independently discovered by two teams. Namdeo et al. (2018) tested the effect of a range of small molecules on mouth-form switch and found that dopamine, tyramine and bisphenol A interfere with the expression of Eu form, whereas Acetyl-CoA induces Eu form. All three former compounds are known substrates of cytosolic sulfotransferases and bisphenol A is known to be a substrate of *C. elegans ssu-1* (Brix et al., 1999; Hattori et al., 2006; Yasuda et al., 2007). Interestingly, there is an expansion of cytosolic sulfotransferases in the *P. pacificus* lineage. *P. pacificus* has 5 *ssu-1* orthologues, which researchers named *sult-1-5*. Two sulfotransferases, *sult-1* and *sult-2*, had an effect on mouth-form in *P. pacificus*, as shown by CRISPR knock-outs and mouth-form screens across different environments (Figure 3). Bui et al. (2018) isolated *eud-1* mutant suppressor lines and mapped the genetic lesion to *sult-1* gene, which they named *seud-1*. *Cel-ssu-1* is expressed in the amphid neurons, as *Ppa-eud-1*, which suggested that they might act on the same substrate in the opposite manner. However, the opposite effect of sulfotransferase *sult-1/seud-1* and

sulfatase *eud-1* does not occur in the same cells in *P. pacificus*, indicating that their interaction is indirect. Surprisingly, *Ppa-sult-1/seud-1* is expressed in pharyngeal muscle cells, supporting its position downstream of *eud-1* switch. Introduction of *sult-1* mutation in *eud-1* mutant background resulted in 10% Eu phenotype. Interestingly, while *eud-1* is unresponsive to *dasc#1*, *Pseudomonas sp.* LRB26 and 10 days of crowding/starvation, *sult-1;eud-1* mutants strongly responded to all three conditions (Namdeo et al., 2018).

Environmental stress induces plastic responses in almost all organisms. One such stress is extremely high temperature. Molecules that mediate this response are called heat-shock proteins and their role has been described in some cases of polyphenism, and even phenotypic assimilation, phenomena in which plasticity predates fixation of a single developmental variant (Rutherford and Lindquist, 1998). In *P. pacificus*, the heat-shock protein *daf-21* regulates the mouth-form by inducing the Eu form (Figure 3). *daf-21* is also involved in the maintenance of the proper morphology of both forms with mutants displaying a wider variation in mouth-form structures than the wild-type comparable to the variation between different species of *Pristionchus* (Sieriebriennikov et al., 2017).

Complex traits like polyphenisms are proposed to evolve in a coordinated manner, and therefore regulated by either a single pleiotropic gene or set of genes in a close proximity of one another, to avoid missegregation caused by recombination (Thompson and Jiggins, 2014). This is the case in some butterfly and ant polyphenisms (Joron et al., 2011; Kunte et al., 2014; Wang et al., 2013). The gene *eud-1* arose from a duplication of an ancestral gene shared with *C. elegans*, *sul-2.2.1* (Ragsdale et al., 2013). Later it was shown that, in *P. pacificus*, these two genes are part of a „supergene" cluster consisting of four genes involved in the regulation of mouth-form. Two other genes are homologues of human N-acetyl-alpha-glucosaminidase (NAGLU), *nag-1* and *nag-2* (Figure 3). Two *nag* genes also are duplicates of an ancient locus of unknown function in other nematodes. However, in *P. pacificus* they modulate the mouth-form polyphenism by promoting the stenostomatous form (Sieriebriennikov et al., 2018).

Very rarely, *P. pacificus* produces intermediate forms that can resemble a pastiche of parts of the two common morphs, with such cases seldom reported in wild-type animals (Sommer et al., 1996). However, a suppressor screen on the *nhr-40* EMS mutant line revealed a downstream nuclear hormone receptor, *nhr-1*, which displays an intermediate phenotype. *nhr-1* mutants have a flapped dorsal tooth characteristic for St, but display a continuous plasticity in the development of its subventral tooth ranging from absence of the tooth to almost wild-type phenotype. *nhr-1* is expressed in the nuclei of several cells in the head, and is most strongly

expressed in pharyngeal muscle cells. Transcriptome analysis revealed that *nhr-1* and *nhr-40* regulate several common target genes expressed in a pharyngeal gland cell, g1D, and are involved in cuticle formation, such as astacins, CAP and chitinases (Sieriebriennikov et al., 2020).

P. pacificus dimorphism is sensitive to several environmental cues, with pheromones (*dasc#1*, *pasc#9*), some bacteria such as *Pseudomonas sp.* LRB26 and agar culturing conditions promoting the Eu form, and magnesium and phosphate salts and liquid culture promoting the St form (Bento et al., 2010; Ragsdale et al., 2013; Namdeo et al., 2016; Werner et al., 2017) (Figure 3). Together, the solid genetic framework described above provides us with the opportunity to investigate the transduction of specific signals from the environment into the genetic switch network.

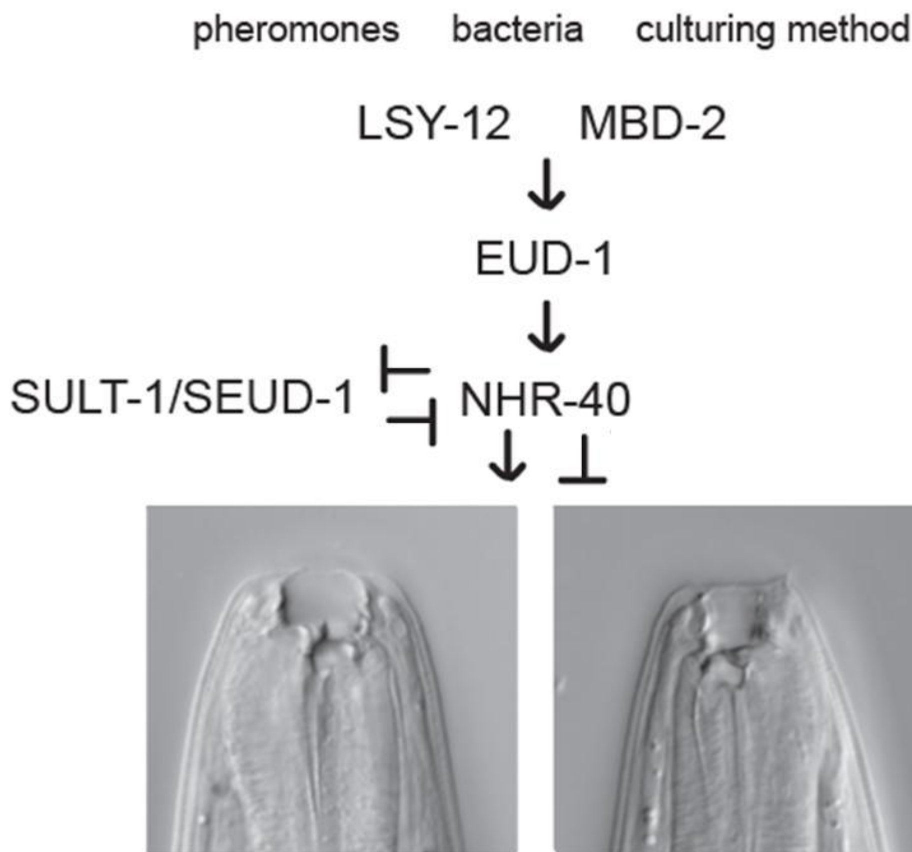


Figure 3: mouth-form dimorphism in model nematode *Pristionchus pacificus*. Environmental signals are integrated through a complex molecular network to produce eurystomaotus (Eu) or stenostomatous (St) form. Stimulation and inhibition arcs indicate phenotypic relationships obtained by epistasis tests, and not molecular regulatory relationships.

1.4 Developmental system drift

The core concept in comparative biology is homology. Aristotle had already noticed how specific traits are shared between different species (Panchen, 1999). During the Renaissance, Pierre Belon conducted the first systematic comparative analysis of bird skeletons (Belon, 1555). In the middle of the 19th century, the German biologist Karl Ernst von Baer noticed that the phylogeny of related species can be more easily determined by looking at the earlier stages of the animal development rather than the later ones, meaning that early stages tend to be more general and reflect properties of families while adult stages tend to be specialized (von Baer, 1828). The term „homology" was coined by Richard Owen, who defined it as the "same organ in different animals under every variety of form and function" (Owen, 1943). Early on, comparative embryologists noticed that traits that appeared homologous in adult morphologies differed in their development. For example, Meckel's cartilage is induced by different tissues in different groups of vertebrates (Wagner and Misof 1993). Development of gene cloning and other molecular techniques in the 1980s enabled researchers to express genes ectopically and directly compare underlying genetic networks in divergent organisms. This decade was marked by the astonishing finding that embryonic patterning in most metazoans is regulated by a conserved Hox gene cluster. Some of these genes were repeatedly recruited in the same developmental pathways that have independently evolved multiple times during the evolution. The *pax6* gene, for example, is a master regulator of eye development in cephalopodes, arthropods and vertebrates although eye structures themselves have evolved separately in these three lineages. This kind of phenomenon is called deep homology (Müller and Wagner, 1996). However, researchers have not always found seemingly homologous structures to be regulated by homologous genes. These incompatibilities were first noticed from crosses between closely related species as increased fluctuating left-right asymmetry in hybrids (Felley, 1980; Graham and Felley, 1985; Leary et al., 1985; True and Haag, 2001). Another early observation came from unexpected bristle formation patterns in *Drosophila* hybrids. Papaceit et al. (1991) show that at least five loci are involved in ectopic sex comb expression on the second and third thoracic segment in hybrids between *Drosophila subobscura/madierensis*, whereas in pure species they are expressed only on the first segment. Following research showed that even highly conserved and fundamental structures such as vulva in nematodes or chordate neurulation have divergent regulatory mechanisms underlying their development. For example, the *P. pacificus* and *C. elegans* vulva is regulated by different, but highly conserved developmental pathways Wnt and EGF, respectively (Zheng et al., 2005a; b; Tian et al., 2008; Wang and Sommer, 2011). Even strains within a single species differ in mechanisms of vulva

development (Kienle and Sommer, 2013). This divergence of regulatory networks underlying highly conserved phenotypic traits is called developmental system drift (DSD) (True and Haag, 2001).

1.5 Aim of the thesis

One of the key questions in developmental biology concerns which kind of molecular mechanisms organisms" sensory systems use to integrate environmental signals into their development. In this thesis, I investigated the receptive molecular pathways responsible for integrating multiple environmental signals in *P. pacificus'* dauer and mouth-form polyphenisms. This was achieved through a candidate gene approach and an unbiased approach using EMS mutagenesis. The candidate gene approach was utilized to investigate the development and roles of primary cilia of sensory neurons in *P. pacificus* nematodes. This project included the investigation of the binding motif and downstream targets of transcription factor *daf-19*/RFX, which belongs to the regulatory factor X transcription family involved in primary cilia formation across animals and whose one-to-one orthologue in *C. elegans* acts as a master regulator of sensory cilia development. We investigated the effects of complete loss of sensory cilia on chemotaxis and social behaviour in *P. pacificus*, as well as dauer development and mouth-form polyphenism. The discovery of mouth-form phenotype in *daf-19*/RFX mutants motivated the investigation of the mouth-form phenotypes of mutants of individual components of sensory cilia previously generated in the laboratory across different environmental conditions. The unbiased approach was used to investigate receptive molecular pathways which integrate temperature signaling into mouth-form developmental switch in *P. pacificus*.

Results

2.1 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.

1.3.2 Synopsys

Phenotypic plasticity is the ability of an organism to change its phenotype in response to alterations in its environment. Phenotypic plasticity is ubiquitous in nature, however, not much is known about the role of phenotypic plasticity as phenomena in ecology and evolution, as well as about the genetic mechanisms behind plastic responses. The tendency of the genetic community to investigate model organisms, which are robust to environmental perturbations, delayed the research of molecular mechanisms which integrate the environment into development. Due to a broad definition, controversies arise when general conclusions are attempted from individual manifestations of plastic responses. This has often led to strong divisions and oppositions within the research community, further hindering empirical research. For example, phenotypic plasticity can be both adaptive and non-adaptive, continuous and discontinuous (also known as polyphenism), conditional and stochastic. Due to the easy culturing, a short generation time of four days, large brood size and amenability to diverse genetic and molecular techniques, including CRISPR-Cas9, *P. pacificus* became one of the most prominent model systems for the investigation of phenotypic plasticity. *P. pacificus* has two polyphenisms: First, a dimorphism of mouth structure, which enables it to exploit different food sources and second, a dauer polyphenism, which enables it to survive adverse environments and disperse *via* beetle hosts. *P. pacificus* presents a unique possibility to investigate contrasting aspects of plastic responses as well as co-evolution between two different polyphenisms. Mouth-form polyphenism in *Pristionchus* is regulated both conditionally (pheromones) and stochastically. Both the mouth form and the dauer polyphenism show continuous changes across environmental stimuli, yet they very rarely form intermediate states between the two forms. While the eurystomatous form is able to predate on other nematodes,

stenostomaotus worms have a shorter generation time when only bacterial food is available, showing that mouth-form dimorphism is adaptive in nature. The main benefit of *P. pacificus* as a model system for phenotypic plasticity is its amenability to experimental tools available for genetic manipulation. This led to identification of the unique switch gene, sulfatase *eud-1,* within only several years of research, and was quickly followed up by a complex network of genetic players which directly or indirectly connect to *eud-1* in mouth-form regulation. *eud-1* is regulated by steroid hormone and small molecule (pheromone) signaling, epigenetic factors (acetyltransferase *lsy-12* and methyl-binding protein *mbd-2* and a long non-coding RNA *lnc-eud-1*), while nuclear hormone receptor *nhr-40* acts downstream to inhibit the carnivorous Eu form. *P. pacificus'* dimorphism was also explored in the context of microevolution (*eud-1* acts in the same fashion among different *P. pacificus* strains) and macroevolution (loss of dimorphism leads to decrease in mouth-form complexity but stronger divergence of the morphology in unimorphic strains as compared to dimorphic, probably as a consequence of character release). Future research in *P. pacificus* and other model organisms will be needed to clarify the role of phenotypic plasticity as agent in ecology and evolution and shine further light on its molecular pathways.

2.1.2. Own contributions

I helped in writing certain sections of the manuscript (approx. 10% of the work).

2.2 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.

2.2.2 Synopsys

Polyphenisms are discrete cases of phenotypic plasticity in which two or more different morphs are produced by different environments. Investigation of genetic and epigenetic mechanisms of polyphenism requires the ability to induce specific morphs, often in large quantities in order to be able to capture the important biological signals. This is challenging in many organisms. Mouth-form dimorphism is *P. pacificus* is one of the best investigated polyphenisms. Two morphs, carnivorous and bacteriovorus, occur in the population in different ratios depending on the environmental conditions. Several environmental factors have been described that promote different morphs, e.g. pheromones, bacteria and sulfate/phosphate salts. However, the precise way of manipulating the amounts of morphs on a large scale was not possible, hindering large scale experiments such as CHIP-Seq. In this paper we found that liquid culture conditions induce the bacterivorus morph, as opposed to highly carnivorous conditions on agar plates. We further tested different parameters of liquid culture condition to investigate their effect on mouth-form development. We found that buffer composition and rotation speed influence mouth-form in an additive way. By finely manipulating one or the other factor we were able to cover the complete span of ratios of the two forms. Finally, we measured the concentrations of previously identified mouth-form related genes, including the master regulator *eud-1* and found that liquid culture conditions act upstream of the described switch network. In summary, we provide the *P. pacificus* community with a robust set of culturing methods for large scale experiments for investigation of mouth-from polyphenism.

2.2.2 Own contribution

I did qPCR measurements of a marker gene (approx. 10% of the work).

2.3 DAF-19/RFX controls ciliogenesis and influences oxygen-induced social behaviors in *Pristionchus pacificus*

Lenuzzi, M. , Moreno, E., Rödelsperger, C., Prabh, N., Witte, H., Roeseler, W., Riebesell, M. and Sommer, R.J., 2018. †

Evolution & Development, 20(6), pp.233-243.

* authors contributed equally

† this paper has been published as Moreno et al., 2018

2.3.1 Synopsys

Primary cilia are complex structures involved in sensory perception in many organisms. However, investigation of molecular mechanisms involved in the formation of the cilia has been challenging since elimination of cilia often results in lethal phenotypes. However, loss-of-cilia mutants are viable in the nematode *C. elegans*, which made it a perfect model organism for the study of cilia and led to in-depth description of the ciliary machinery. However, comparative studies in other nematode species are rare. One recent study showed overlap but also differences in the contribution of ciliary components to social behaviour in *P. pacificus*. Primary cilia formation is dependent on the transcription factor of the „regulatory factor X" (RFX) family. In *C. elegans*, the sole member of this family, *daf-19*/RFX, is required for the formation of primary cilia of sensory neurons, a main chemosensory organ of this nematode species. In this paper, we tested the conservation of the *daf-19*/RFX in *P. pacificus*. We performed CRISPR knock-out of one-to-one ortholog of *Cel-daf-19* in *P. pacificus* PS312 and obtained two frame-shift mutants. *daf-19* mutants in *P. pacificus* showed defects in chemotaxis and oxygen sensation. Interestingly, *Ppa-daf-19* mutants did not show social behavior, unlike the downstream structural mutants of cilia. Electron microscopy sections and dye-filling experiments revealed that *daf-19* is necessary in *P. pacificus* for proper cilia formation. We showed further *via* RNA-Seq that *Ppa-daf-19* regulates the expression of the structural components of the cilia as its *C. elegans* ortholog. RFX family transcription factors bind to a highly conserved X-box motif sequence. We identified in silico a conserved X-box motif in the promoter region of *P. pacificus* genes coding for structural components of the cilia. Disruption of the motif by CRISPR-Cas9 mutagenesis in *dyf-1* resulted in the dye-filling phenotype equivalent

to the one observed in the mutants, which harbor frame-shift mutations in the coding region. Interestingly, we found that *Ppa-daf-19* mutants were not dauer constitutive as in *C. elegans*, but rather seem slightly defective in dauer formation. In summary, our study shows conservation of *daf-19* in cilia development and its divergence in dauer regulation.

2.3.2 Own contribution

Dr. Moreno and I did together dauer and chemotaxis assays and screens, dye-filling, manual motif search, CRISPRs (not injections), RNA-Seq library, data analysis, figure preparation and manuscript draft. I did confocal microscopy imaging and RACE. I contributed to approx 40% of the work.

2.4 Cilia drive developmental plasticity and are essential for efficient prey detection in predatory nematodes

Moreno, E., Lightfoot, J.W., Lenuzzi, M. and Sommer, R.J., 2019.

Proceedings of the Royal Society B, 286(1912), p.20191089.

2.4.1 Synopsys

Polyphenisms depend on environmental sensing in order to express the appropriate phenotype. Nematodes sense their environment through primary cilia of sensory neurons. In this work we investigated the role of cilia in regulating the expression of predatory eurystomatous (eu) and bacteriovorous stenostomatous (st) morph in *Pristionchus pacificus*. We used laboratory CRISPR-Cas9 generated mutants of structural components of the cilia representing all major protein subcomplexes, as well as transcription factor *daf-19/*RFX, a master regulator of cilia formation in *P. pacificus* to investigate the role of sensory cilia in mouth-form polyphenism. IFT B protein complex and kinesins are involved in anterior transport along the cilia, ITF-A complex and dyneins are associated with retrograde transport and BBSome serves as a bridge between the two complexes. First, we screened the mouth-form phenotype of the mutants of two components of IFT-B complex (*osm-1* and *dyf-1*), kinesin dimer (*osm-3*;*klp-20*), three components of IFT-A (*ifta-1*, *che-11*, *dyf-2*), dyneins (*che-3* and *xbx-1*) and BBSome protein *osm-12*. The mutants were grown on two Eu-inducing conditions (agar and *Pseudomonas sp.* LRB26) and one St-inducing condition (liquid culture). We found contrasting roles of different ciliary components. Interestingly, most proteins are positively regulating the induction of the carnivorous eu morph. *daf-19*/RFX produced more St animals than the wild-type, both on agar and LRB26. Two investigated mutants of the components of the anterior transport machinery, IFT-B protein complex subunits *osm-1* and *dyf-1* had strong St phenotype on the agar plates and on LRB26. Unlike in *C. elegans*, kinesin motor proteins are redundant in *P. pacificus*. The double mutant *osm-3;klp-20* was highly St on agar, whereas on LRB26, the difference from the wild-type was not significant. Higher st:eu phenotype of IFT-A protein complex subunits *ifta-1* mutant was significantly different from that of wild-type animals only on agar, while *che-11* mutants had significantly higher st:eu ratio in both conditions, agar and LRB26. Interestingly, two dynein mutants showed opposite effects on mouth-form formation. While *che-3* mutants had significantly lower numbers of eu animals on agar and LRB26, *xbx-1* mutants had significantly higher levels of eu animals in liquid culture. Mutants of

the BBSome component *osm-12* were highly eu in liquid culture. Second, we investigated the role of cilia in prey recognition. *P. pacificus* PS312 eu form predates nematodes, including other *P. pacificus* strains. However, it has a specialized self-recognition mechanism which prevents worms from feeding on the members of their own strain. We investigated whether predatory behavior is regulated by cilia. We used two highly st mutants *daf-19* and *osm-1*, and two highly eu mutants *che-3* and *osm-12*. While all mutants had no problem with self-recognition, they were all defective in predation on *C. elegans* L1 larvae. Our results confirm expectation that cilia are crucial for normal expression of two morphs, and are required for both morphology and behaviour.

2.4.2 Own contributions

I did mouth-form phenotyping of wild-type and mutants on agar and bacteria. My contribution to this paper is approx. 30%.

2.5. Co-option and developmental system drift in sensory mechanisms of developmental plasticity in nematodes

Lenuzzi, M., Rödelsperger, C., Riebsel, M., Witte, H., Moreno, E., Sommer, R. J., *in prep.*

2.5.1 Synopsys

Temperature is one of the major abiotic signals in the development. However, very little is known about the receptive molecular pathways through which temperature modulates the development. Mouth-form dimorphism of *P. pacificus* nematodes (omnivorous and bacteriovorus form) is an established model system for the investigation of genetics of developmental plasticity. *P. pacificus* is simple to maintain in the laboratory culture, has a short generation cycle and is a self-fertilizing hermaphrodite. Multiple molecular biology and genetics methods, including CRISPR-Cas9 and EMS mutagenesis, have been successfully applied in *P. pacificus.* This work explores the receptive molecular pathways which integrate high temperatures into polyphenic switch in mouth-form dimorphism.

The general reaction norm of mouth-form dimorphism (laboratory strain RS2333 and 10 natural variants across the temperatures 10-28°C) resembles a bell-shaped curve, with mostly omnivorous at intermediate and more bacterivorus towards extreme temperatures. Interestingly, reference strain RS2333 showed low plasticity at a high temperature range, possibly due to domestication effect. Therefore, we established the natural isolate RSA635, showed strong and robust increase in bacteriovorus form towards 28°C, as a model system for the investigation of the receptive pathways of high temperature integration into mouth-form dimorphism. Eleven mutants were obtained by ethyl methyl-sulfonate mutagenesis which lost the mouth-form plasticity at high temperatures. The mapping of two mutants revealed the role of *daf-11*/guanylyl cyclase and its localizer into sensory cilia *daf-25*/Ankmy2 in the induction of the bacteriovorus morph. Candidate approach identified the *tax-2*/cGMP-gated ion channel as a possible downstream target of *daf-11*-regulated cGMP signalling.

Besides mouth-form polyphenism, *P. pacificus* has also another, dauer, polyphenism. Dauers are a highly resistant type of larva adapted for survival in stressful conditions. *P. pacificus* shares dauer polyphenism with other nematode species, such as model nematode *C. elegans*. Like mouth-form polyphenism, dauer polyphenism in *C. elegans* is temperature-sensitive: more dauers form at high temperatures. This parallel inspired us to check whether dauer polyphenism is temperature-sensitive in *P. pacificus*. Dauer assays at 20 and 28°C showed that *P. pacificus*

wild type dauers are also temperature-sensitive. Interestingly, *daf-11* and *tax-2* mutants had opposite dauer formation defects compared to the mutants in *C. elegans;* while dauer constitutive in *C. elegans*, they are dauer defective in *P. pacificus*. Whether developmental system drift in dauer regulating pathway is the consequence of co-option of *daf-11* and *tax-2* into the regulation of mouth-from polyphenism remains an interesting hypothesis for the future research.

Comparative analysis of *daf-11* expression between *P. pacificus* and *C. elegans* reveals both conservation and divergence of ancestral pattern. In both *P. pacificus* and *C. elegans*, *daf-11* is expressed in sensory neurons and *daf-11* is probably localized in sensory cilia in *P. pacificus* as its one-to-one ortholog in *C. elegans*. While the expression pattern of *daf-11* in individual neurons overlaps between the two species, it is not identical. Perhaps differences on the phenotypic level can be explained by different molecular contexts of *daf-11* between the two related nematodes.

This study advances the understanding of receptive genetic pathways of developmental plasticity.

2.5.2 Own contribution

I did partial project conceptualization, all the assays and phenotyping, EMS mutagenesis, most of CRISPR-Cas-9 injections and sequencing (except *tax-2*), BSA, transgenesis, statistical analysis, manuscript draft and figure preparation (90% of the work).

2.6 General results and discussion

The genetics of developmental plasticity have only begun to be elucidated. This study utilized the amenability of polyphenic *P. pacificus* nematodes to genetic research to study the receptive pathways of developmental plasticity.

A decade of research of mouth-form (summarized in 2.1) and dauer polyphenism of *P. pacificus* has led to the discovery of a complex switch network, together with the associated environmental conditions affecting the switch. However, the receptive pathways which integrate the environmental signals into the switch network remain unclear. Which tissues sense the environment? Which kinds of molecules and developmental pathways are involved? How conserved are these pathways at the micro- and macroevolutionary level? Below, I will summarize and discuss the results through this umbrella of important questions at the heart of research on developmental plasticity.

2.6.1 Tissue: Role of sensory cilia in the regulation of developmental plasticity

This work shows that the sensory cilia are responsible for integrating environmental signals into mouth-from and dauer polyphenism. This chapter includes an investigation of the conservation of the regulation of sensory cilia development by RFX family transcription in *P. pacificus* (2.6.1.1), and the investigation of the role of sensory cilia in mouth-from and dauer polyphenism (2.6.1.2. and 2.6.1.3).

2.6.1.1 Sensory cilia are a major chemosensory organ and have highly conserved development among nematodes

The first empirical evidence of the role of the RFX family of transcription factors in the ciliogenesis was the identification of the role of *daf-19*/RFX in primary cilia development in *C. elegans* (Swoboda et al., 2000). Follow-up research discovered the highly conserved role of RFX transcription factors as one of the major regulators of ciliogenesis across eukaryotes (Thomas et al., 2010). Genetic and molecular biology analysis, imaging and phenotyping showed that the one-to-one ortholog of a sole master regulator of cilia formation in *C. elegans*, *Ppa-daf-19*/RFX, is a master regulator of sensory cilia formation in *P. pacificus* (chapter 2.3). Briefly, dye-filling experiments showed dye uptake defects in amphid neurons and transmission electron microscopy (TEM) revealed closed amphid channels without cilia in mutant adults and

strong defects of the cilia of cephalic and labial neurons. RNA-Seq comparison of mutant and wild-type showed strong down-regulation of structural components of cilia in mutant embryos. Moreover, the highly conserved transcription factor binding motif (X-box motif) is also found in *P. pacificus* ciliary genes. A CRISPR-Cas9 knock-out of the motif replicates the phenotype of exonic mutants, as shown on the example of *dyf-1* gene (Swoboda et al., 2000; Piasecki et al., 2010). *Ppa-daf-19* mutants showed defects in chemotaxis to bacteria and had severe defects in oxygen sensation, further demonstrating the major role of sensory cilia in chemosensation among nematodes. Therefore, regulation of ciliogenesis by RFX transcription factor is conserved in *P. pacificus*.

2.6.1.2 Sensory cilia integrate signals into mouth-form dimorphism

The role of sensory cilia in perception of signals for mouth-form dimorphisms has been discovered both through a candidate approach (2.6.1.2.1) and an unbiased mutagenic approach (2.6.1.2.1).

2.6.1.2.1 Candidate approach

Strong disruption of cilia function is often lethal for higher organisms, due to their crucial role in development and tissue homeostasis (Feng et al., 2009). Contrary, in *C. elegans*, complete disruption of cilia through removal of the master regulator *daf-19*/RFX results in a myriad of sensory defects, but not lethality. This made *C. elegans* a perfect model for the research of mechanisms of primary cilia and led to a comprehensive description of regulatory and structural components of cilia (Inglis et al., 2018). We drew from this rich literature to perform candidate screens in *P. pacificus* for molecules involved in polyphenism regulation.

Sensory cilia play a vital role in the regulation of mouth-form switch (chapter 2.4). The cumulative loss of cilia in *daf-19* null mutants leads to severe failure in the induction of eu morph across conditions (see chapter 2.3). This inspired further research of the role of individual components of cilia on mouth-form. Furthermore, the CRISPR/Cas9 mutants of ciliary proteins covering all IFT subcomplexes were already present in the lab (Moreno et al., 2017). The screens across two eu- and one st-inducing (chapter 2.2.) conditions showed that anterograde transport through the IFT-B subcomplex and kinesins is the most important for the induction of eu morph, whereas retrograde transport through the IFT-A subcomplex has a moderate role in eu induction (reviewed in Pederson and Rosenbaum, 2008). Anterior transport

is crucial for cilia assembly and maintenance in *Chlamydomonas*, and its loss leads to very small or absent cilia. Conversely, retrograde transport plays a role in receptor turnover and is not needed for cilia assembly (Ishikawa and Marshall, 2017). Therefore, stronger defects in cilia formation in the mutants defective in anterograde transport might explain the stronger phenotype. Interestingly, mutants of two molecules of the dynein complex had contrasting phenotypes. The dynein motor subunit *che-3* mutant was highly st, while the dynein light intermediate chain *xbx-1* mutant was highly eu (Wicks et al., 2000; Schafer et al., 2003). It appears that the two dynein chains perform distinct functions in the context of the mouth-form dimorphism. A single BBSome component that was phenotyped, *osm-12*, strongly induced st form. The BBSome subcomplex keeps two kinesin molecules together and is directly involved in receptor trafficking in and out of cilia (Ou et al., 2005; Wingfield et al., 2018). It is not clear which, if any, of these functions is related to high eu mutant phenotype.

Some bacteria, such as *Pseudomonas sp.* LRB26, are potent inducers of eu-form. The comparison of mutant phenotypes between standard laboratory condition (agar + *E.coli*) and strongly eu-inducing condition (agar + eu-inducing *Pseudomonas sp.* LRB26) might tell us something about the pathways of integration of eu-inducing signals from bacteria. The comparison of eu:st ratio in *daf-19* mutants between the two conditions suggest that *Pseudomonas sp.* LRB26 is sensed both by the cilia, but also downstream or in parallel with cilia. Perhaps it is also sensed by the intestine and the signallings from the intestine and cilia are integrated further downstream in the mouth-form switch. The role of cilia can perhaps be attributed partially to receptors trafficked by *che-11,* which is defective in the sensing of *Pseudomonas sp.* LRB26. *Cel-che-11* are chemotaxis defective and perhaps chemosensation plays an active role in eu induction on this bacterium, although it is possible that some other sensory defects present in *che-11* mutants are responsible for the mutant phenotype (Bargmann at al., 1993). Interestingly, *dyf-1* has an equal eu:st ratio in both standard conditions and NGM agar + *Pseudomonas sp.* LRB26, indicating that it plays a strong role in eu-induction by *Pseudomonas sp.* LRB26. Either it is directly involved in the trafficking of the receptor involved in the sensing of eu-inducing bacterium, or it causes defects of cilia involved.

Mouth-form is a complex phenotype consisting of morphology and behaviour. The predatory eu form displays predation-associated behaviors, such as biting and sucking, whereas the st form doesn't show any predatory intentions (Wilecki et al., 2015). All phenotyped ciliary mutants, including *daf-19*, display substantially lower rates of predation on *C. elegans* larvae. However, they all showed complete self-recognition. Either some redundant activity of cilia is still present in *daf-19* mutants, or self-recognition is regulated by other tissues. Perhaps

the remnants of cephalic and labial cilia perform the self-recognition function in *daf-19* mutants (Moreno, Lenuzzi et al., 2018).

2.6.1.2.2 Unbiased approach

Temperature-dependent reaction-norms are ubiquitous and the literature on them is substantial. Additionally, in the context of the recent global increase in temperature, understanding plastic responses to temperature changes has gained further importance. Chapter 2.5 shows that the general reaction norm of *P. pacificus* mouth-form resembles a bell-shaped curve with maximum eu:st ratio at mid temperature range, based on the screen of 10 natural variants isolated from various locations within tropical and moderate climate ranges. Variation in the shape of individual curves implies differences in the genetic regulation of plasticity (or G x E interactions) in individual strains. The bell shaped reaction norms across temperature gradients are commonly found in nature and some researchers propose that bell shaped responses might be a fundamental property of temperature regulated reaction norms (Graves, 2008). Interestingly, *P. pacificus* displays a bell shape reaction norm across salt gradient, indicating that increase in st might be a general strategy in the conditions of abiotic stress (chapter 2.5). Perhaps faster development of st form poses a selective advantage under harsh conditions (Serobyan et al., 2013). Additionally, from my personal observation, eu worms reared and fed at high temperatures show lower killing rates of *C. elegans* larvae in comparison to one's reared at normal temperatures. The shape of the mouth structures gets more variable towards extreme temperatures and the deformities in shape of eu mouth-form perhaps lead to lower killing efficiency (Sieriebriennikov et al., 2016). Therefore, the low killing ability might not be able to compensate for the cost of the longer developmental time, leading to the selection against the development of the eu mouth-form at higher temperatures. However, precise observations and associations of this behaviour and morphologies and finally selective (dis-) advantages awaits future research.

The mapping of two EMS-derived mutants which failed to induce st at high temperatures revealed a crucial role of cGMP signaling pathway of sensory cilia in the genetic regulation of mouth-from plasticity. Two analyzed mutants mapped to one-to-one orthologs of *C. elegans daf-11* and *daf-25* genes. Membrane-bound receptor guanylyl cyclase (GC) *daf-11* is involved in chemotaxis and temperature sensing in *C. elegans* and it interacts with Hsp90 heat-shock protein *daf-21* (Birnby et al., 2000). *Cel-daf-25* is an Ankyrin Repeat And MYND Domain Containing 2 (Ankmy2)-domain containing protein needed for proper localization of *Cel-daf-11* into primary cilia of sensory neurons and mutants display similar defects to the ones of *Cel-daf-*

11 mutants. The role of Ankmy2 proteins seems to be evolutionarily conserved; for instance, a homolog of *daf-25* in the retina of mice binds to mouse retinal GC1 (Jensen et al., 2010). Similarly, cGMP signaling regulates behavioural polyphenism across insects. cGMP-dependent protein kinase (PKG) *foraging* regulates temperature, an oxygen-sensitive rover-sitter phenotype in *D. melanogaster* and foraging behaviour in eusocial species and might be involved in the regulation of dispersal polyphenism in locusts (Anreiter and Sokolowski, 2019).

In *C. elegans*, the cilia-located cyclic nucleotide-gated (CNG) Ca2+-channel *tax-2/tax-4* has been proposed as a downstream target of *daf-11* signalling (Birnby et al., 2000, Ailion and Thomas, 2000). This motivated me to investigate the role of *Ppa-tax-2/tax-4* in high-temperature induction of st. *Ppa-tax-2* CRISPR-Cas-9 mutants were highly eu on both 20°C and 27°C. Therefore, cGMP signalling from *daf-11* potentially targets *tax-2/tax-4* channel, however, there is also a possibility that *tax-2/tax-4* act independently of *daf-11* signalling. *tax-2/tax-4* is itself an important temperature sensor involved in thermotaxis behaviour in AFD neurons (Ramot et al., 2008). However, research in *C. elegans* showed minor overlap between the pathways involved in thermotaxis and dauer regulation, including the minor role of AFD in dauer induction.

cGMP signalling regulates thermotolerance in insects and vertebrates (Dawson-Scully et al., 2007; Robertson and Sillar, 2009; Armstrong et al., 2010). Whether the st morph displays higher thermotolerance than the eu morph remains to be tested.

Together, the results discussed under 2.6.1.2.1 and 2.6.1.2.2 show that environmental signals which regulate mouth-form dimorphism in *P. pacificus* are sensed through primary cilia of sensory neurons.

2.6.1.3 Sensory cilia integrate signals into dauer polyphenism

In *C. elegans*, cumulative loss of cilia through *daf-19*/RFX mutation leads to daf-c phenotype (Perkins et al., 1986; Malone and Thomas, 1994; Sentiand Swoboda, 2008). However, no dauers were observed in *Ppa-daf-19* mutants in standard laboratory conditions. Crowding/starvation and exposure to high temperature for 12 days induced dauers in both wild type and *daf-19* mutants to a similar extent. Therefore, in *P. pacificus*, either sensory cilia have no role in dauer formation or the pathways of sensory cilia that integrate both dauer promoting and dauer inhibiting signals are equally represented. The former is highly unlikely, since active dauer pheromone was isolated from *P. pacificus*, which is most likely perceived by cilia, based

on the homology in *C. elegans* and the role of *P. pacificus* cilia in chemotaxis (Bose et al., 2014; chapter 2.3). Additionally, sensing of insect pheromone depends on OBI-1 protein, which localises around amphid cilia (Cinkornpumin et al., 2014).

In *C. elegans, daf-11* and *tax-2* are daf-c with the strongest daf-c phenotype at high temperatures. In the present study, their roles in dauer polyphenism in *P. pacificus* were tested. Dauer assays showed that *Ppa-daf-11* and *Ppa-tax-2* both act as dauer inducers at both low (20°C) and high (27°C) temperatures. *tax-2* mutants have an especially strong phenotype and only a single dauer larva was scored (at 20°C). Interestingly, while in wild-type dauer production increased with temperature, in *daf-11* mutants it slightly decreased. Although this decrease in dauer numbers in *daf-11* at 27°C was minor, it was consistent across all three replicates. These results can perhaps be explained by different pathways regulating dauer formation on low and high temperatures and their different dependence on *daf-11*. Similarly, Hid phenotype in *C. elegans* revealed the existence of alternative pathways which regulate dauer formation on high temperatures. *Cel-tax-2* mutants show slightly daf-c phenotype on temperatures lower than 26.6°C and become non-linearly more daf-c on the temperatures above (Ailion and Thomas, 2000).

2.6.1.4 Conclusion

The results presented in the chapter 2.6.1 show that the development of sensory cilia in *P. pacificus* depends on a highly conserved master regulator *daf-19*/RFX and that the sensory cilia play a vital role in environmental signal integration in the mouth-form and dauer polyphenisms. However, there remain many uncertainties as to the precise stimuli which induce one form over the other in the investigated conditions. It is not clear why nematodes are highly eu on agar and highly st in liquid culture. The complex culturing conditions requiring bacterial food source and complex culturing medium hinder the research of specific environmental agents. In the chapter 2.5 it is not possible to exclude indirect effects of temperature through bacteria or pheromones. For example, high temperatures sensitize *C. elegans* to pheromones, but it also works in a pheromone-independent way to induce dauer formation (Ailion and Thomas, 2000). Possibly the same is the case in *P. pacificus*, and several signals interact synergistically to produce the adaptive phenotype. Future research will be needed to supplement the findings in this thesis to expand our understanding of specific inductors. An understanding of specific contributions of particular molecules and tissues across conditions would perhaps also help identifying the ligands and signals.

2.6.2 Evolution of polyphenism

2.6.2.1 Mouth-form polyphenism evolved through co-option of signalling pathways in sensory cilia

How organisms acquire novel traits is one of the central questions in evolutionary biology. The work in this thesis shows that mouth-form polyphenism in *P. pacificus* evolved through general co-option of sensory cilia. IFT machinery of sensory cilia has been widely co opted in the regulation of both eu and st mouth-form across different conditions (chapter 2.4), while highly conserved cGMP signaling is co-opted into high temperature induction of st and dauer (chapter 2.5). While the previous research, based on a candidate approach, suggested an important role of co-option in the evolution of mouth-form dimorphism, this work underlines the importance of co-option through a thorough candidate screen and unbiased approach (Bento et al., 2010; Kroetz et al., 2012, Sieriebriennikov et al., 2018). The evolution of novel complex traits requires many genes; thus, having available sensory molecules and signalling pathways at hand could substantially reduce the time required to evolve. Indeed, research in insects showed prevalent co-option of hormonal signalling into polyphenism regulation (Nijhout, 1999). Furthermore, the hedgehog signaling (hh) module of primary cilia has been co-opted into wing patterning in seasonally polyphenic butterflies and beetle horn polyphenism (Keys et al., 1999; Kijimoto and Moczek, 2016).

One phenotypically plastic trait can perhaps facilitate the evolution of another plastic trait by providing the genetic toolkit for plasticity (Bradshaw, 1965). Perhaps the dauer polyphenism facilitated the evolution of mouth-from polyphenism through co-option, since many of the molecules co-opted into mouth-form dimorphism had a role in dauer polyphenism in *P. pacificus, C. elegans* or both. For example, the steroid hormone *daf-12* and the protein kinase *egl-4* have been co-opted into mouth-form plasticity and are both involved in the older dauer polyphenism in *C. elegans* and *P. pacificus* (Bento et al., 2010; Kroetz et al., 2012). The highly conserved molecules discovered in this research probably seem to predate both polyphenisms, but it nonetheless appears plausible to suspect that the dauer polyphenism provided a stimulating context facilitating the evolution of the novel mouth-form polyphenism. The extend of the downstream pathways shared between the two polyphenisms in the context of temperature remains elusive.

2.6.2.2 DSD in receptive pathways of dauer polyphenism

Both *C. elegans* and *P. pacificus* produce more dauer larvae in stressful conditions, such as crowding/starvation or high heat (Hu et al., 2007; chapter 2.5). However, molecular pathways which regulate dauer reaction norm have substantially diverged between the two related nematodes. As mentioned earlier in 2.6.1.3, the cumulative effect of cilia loss leads to daf-c phenotype in *Cel-daf-19* mutants, while *Ppa-daf-19* mutants produced wild-type dauer ratios under the same conditions (Perkins et al., 1986; Malone and Thomas, 1994; Sentiand Swoboda, 2008; chapter 2.3). Furthermore, *daf-11* and *tax-2* mutants have contrasting dauer phenotypes in *C. elegans* and *P. pacificus*. The DSD in the sensory system is probably a result of the diverse ecological function of the dauer stage between the two species. Interestingly, comparative research in *Caenorhabditis* nematodes suggested considerable DSD in dauer formation within the genus. Phenotyping of one-to-one ortholog RNAi knock-down lines between closely related *C. elegans* and *C. briggsae* have found that 7% likely diverged in function (Verster et al, 2014).

Similar divergence has been observed in the pathways downstream of dauer switch or the ´dauer phenotype execution´ pathways (Sinha et al., 2012). Interestingly, the major switch for dauer induction, the nuclear receptor *daf-12*, is conserved between *C. elegans* and *P. pacificus* (Ogawa et al., 2009). The observations of molecular pathways and environmental signals across insect species led some researchers to propose the higher evolvability of pathways upstream and downstream of switch (Bopp et al., 2014; Trible and Kronauer, 2017). Mayer et al., 2015 found recruitment of an orphan gene into dauer regulation upstream of *Ppa-daf-12*. Observations from chapter 2.3. and 2.5 provide further support to the hourglass-like evolutionary model of developmentally plastic phenotypes proposed by the research community working on insects (Ogawa et al., 2009; Sinha et al., 2012).

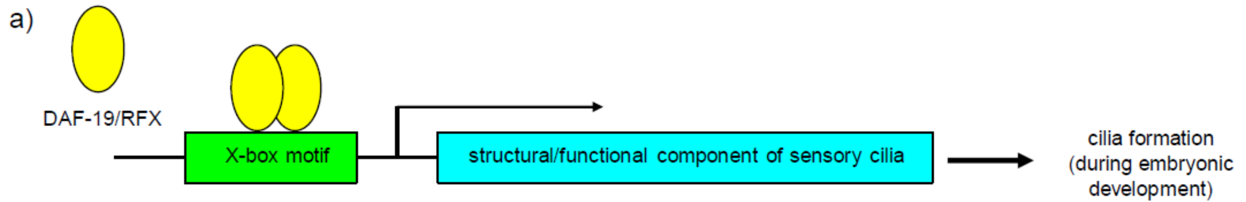
The extensive developmental system drift in dauer formation could, to some extent, be a consequence of co-option of receptive pathways into the novel mouth-form polyphenism. It has been proposed that pleiotropic genes frequently switch their function due to selective pressures imposed by novel phenotypes into which they are co-opted (Johnson and Porter, 2007). Perhaps the evolution of the novel mouth-form polyphenism, other reaction norms of dauer polyphenism or other plastic phenotypes lead to different selective pressures on the described pleiotropic factors and their divergence.

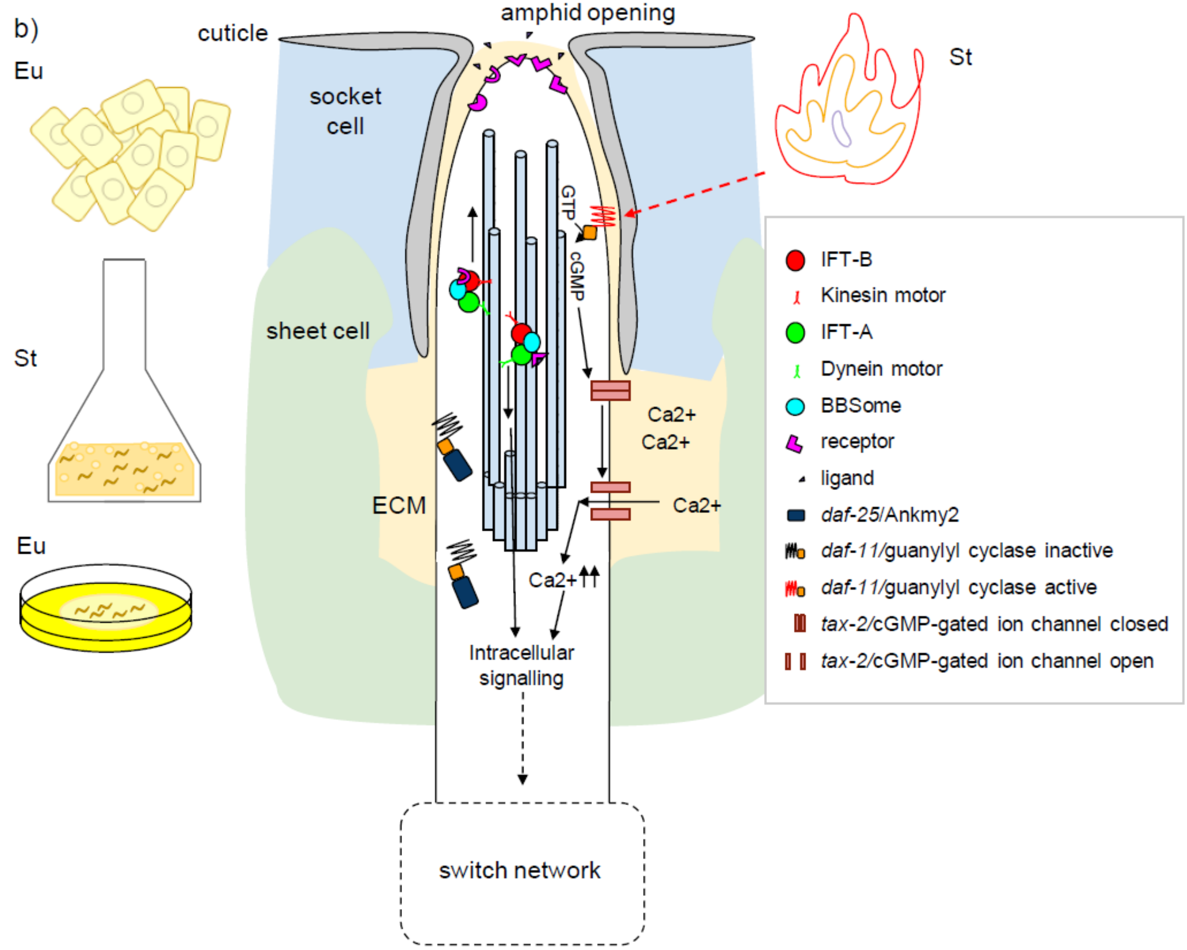
2.6.3. Conclusion

In the present study, it is shown that the primary cilia of sensory neurons show a highly conserved development and are responsible for integrating several environmental signals into developmental plasticity across nematodes. The prevalent co-option of molecular pathways in polyphenism evolution encourages further candidate screening for regulators of polyphenisms in other phyla and gives research in nematodes a valuable predictive power in the research of polyphenism genetics. However, prevailing DSD reveals the importance of unbiased approaches and studying more than one species comparatively, even within closely related groups (True and Haag, 2001). This work expands the knowledge of the receptive pathways of developmental plasticity and paves the way for the further studies of the mechanisms of signal integration into mouth-form dimorphism in *P. pacificus*, providing a resource for comparative genetics research of developmental plasticity in other organisms.

2.6.4. Graphical summary

- on the following page


a)
DAF-19/RFX
X-box motif
structural/functional component of sensory cilia
cilia formation
(during embryonic
development)


b)
Eu
St
Eu
cuticle
amphid opening
socket
cell
sheet cell
ECM
GTP
cGMP
Ca2+
Ca2+
Ca2+
Ca2+↑↑
St
Intracellular
signalling
switch network
IFT-B
Kinesin motor
IFT-A
Dynein motor
BBSome
receptor
ligand
daf-25/Ankmy2
daf-11/guanylyl cyclase inactive
daf-11/guanylyl cyclase active
tax-2/cGMP-gated ion channel closed
tax-2/cGMP-gated ion channel open

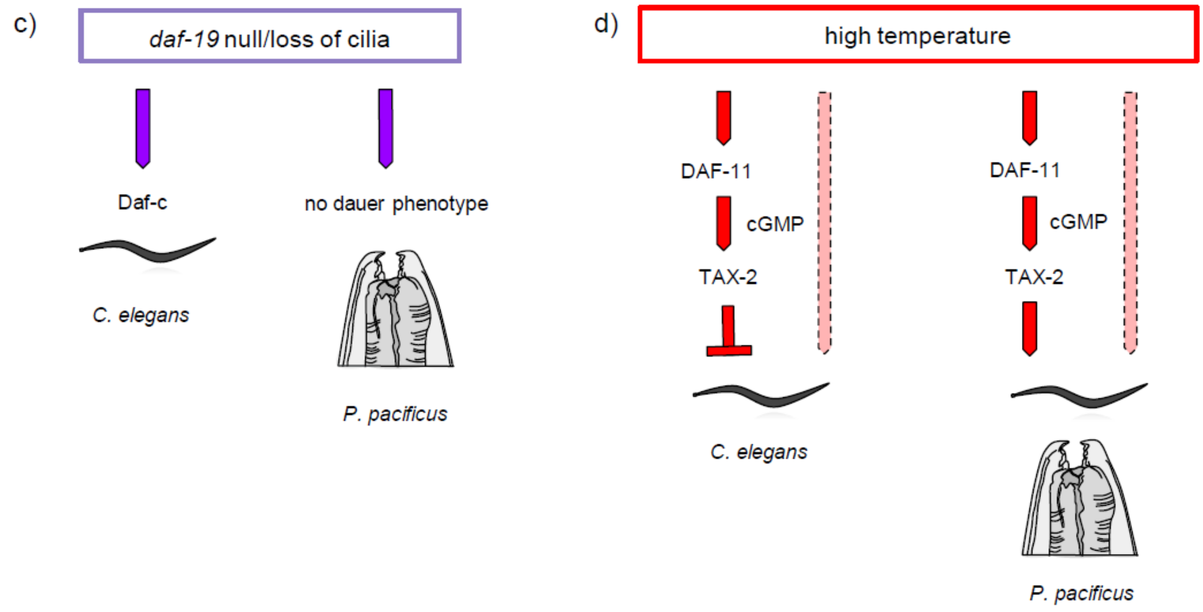

c)
daf-19 null/loss of cilia
Daf-c
C. elegans
no dauer phenotype
P. pacificus
d)
high temperature
DAF-11
cGMP
TAX-2
C. elegans
DAF-11
cGMP
TAX-2
P. pacificus

Figure 4: graphical summary of the results in this thesis. a) *daf-19*/RFX is a master regulator of sensory cilia formation in *P. pacificus*, as in *C. elegans*; b) Schematic representation of primary cilia with major subcomplexes (on the tubulin doublets in the middle) and cGMP pathway (left and right membrane) which play a role in mouth-form polyphenism regulation as described in this thesis. The environments are shown with the respective mouth-form which they induce. Temperature is integrated through the *daf-11*/guanylate cyclase and cGMP pathway, while signals on the left require structural and functional components of cilia (kinesin, IFT-B, IFT-A, dynein, BBSome). c) and d) show co-option of sensory cilia pathways into mouth-from polyphenism in *P. pacificus* and DSD within ciliary pathways in the regulation of dauer polyphenism between *C. elegans* and *P. pacificus*. While *daf-19* mutants in *C. elegans* are Daf-c, they do not show dauer phenotype in *P. pacificus*, but they are defective in eu morph production (c). In both *C. elegans* and *P. pacificus*, high temperatures induce dauer formation. High temperature is sensed by *daf-11*/guanylyl cyclase in both *C. elegans* and *P. pacificus*. While in *C. elegans daf-11* suppresses dauer formation, in *P. pacificus* it induces dauer formation and has acquired a novel role in the positive regulation of st mouth-form at high temperatures (d).

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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.

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*authors contributed equally

Acknowledgements

I would like to thank my supervisor Prof. R. J. Sommer for providing me with the opportunity to work in a highly stimulating environment and for his guidance!

I am very thankful to Dr. Kohta Yoshida for training my logical and creative thinking through scientific discussions within formal and informal environments, for the help he provided me aptly with computational and statistical analysis and story development, and the most for being a dear and caring friend.

I am deeply grateful to my second supervisor Prof. Oliver Bossdorf and TAC committee members Dr. Oliver Weichenrieder, Prof. Gáspár Jékely and Dr. Estienne Swart who generously advised me on my project and career from the depth of their scientific and academic experience.

I was extremely lucky to have amazing and helpful experts, such as Dr. Christian Feldhaus, who introduced me to the magic world of microscopy with his fun but serious and always ready to help disposition, and Dr. Christian Rödelsperger, who guided me through the forests of codes and who shared with me generously several bioinfo pipelines he built.

I would also like to thank Dr. Eduardo Moreno, for sharing my passion for integration of environmental signals into genetic switches which resulted in a more than a year long collaboration and several publications, for his help with scientific talk preparations and the weakly yoga classes which allowed me to regain stability during times of high pressure!

I am also thankful to Dr. Vladislav Susoy, who guided me as a senior student through my first year of PhD with his enormous expertise and passion for science.

I am very, very thankful to the lab technicians Heike, Hanh, Walli and Metta for the direct help with the experiments, as well as day to day support and fun talks in the kitchen. I am also thankful to lab technicians Gabi, Thobias and Dorothee, as well as technical from the whole institute, for their advices and help with experiment conduct.

To my parents, Nada and Ivan, and my sister Irena, I will forever be indebted for their enormous emotional and financial support, during my whole life and education, and I wish that my thesis makes you happy and proud of my (our) achievement.

I am very thankful to Lars for the translation of the summary into German and for refining my academic English in the rest of the thesis, as well as for the everyday inspiration, encouragement and beauty.

I would like to thank Prof. Xin Yu for his mentorship over the years, I will always cherish the memories of our strolls, the stories and advices he generously shared.

I am very thankful to Dr. Heiko Schmidt for his input to my thesis, as well as for being a wonderful flatmate.

Many, many thanks to the whole lab, to the colleagues who helped me to grow as a scientist, who taught me experimental and computational methods, who helped me by engaging in my project during lab meetings, but also to those who taught me how to protect myself and stand up for myself when necessary.

An enormous thank you goes to all my dear friends from the lab and outside the lab - and here I wish to thank my family again - for being there for me, energizing me, and listening to me discuss my research patiently and with curiosity, improving the flow and vision of the story I am trying to tell.

Finally, I would also like to thank all of the people I have met and seen at the institute, conferences and symposiums, who shared their experiences and scientific passions with me, in this way inspiring me and broadening my horizons!

Appendix

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Review

Cite this article: Sommer RJ, Dardiry M, Lenuzzi M, Namdeo S, Renahan T, Sieriebriennikov B, Werner MS. 2017 The genetics of phenotypic plasticity in nematode feeding structures. *Open Biol.* **7**: 160332. http://dx.doi.org/10.1098/rsob.160332

Received: 12 December 2016
Accepted: 10 February 2017

Subject Area:
developmental biology/genetics

Keywords:
phenotypic plasticity, *Pristionchus pacificus*, switch genes, nuclear hormone receptors, epigenetics

Author for correspondence:
Ralf J. Sommer
e-mail: ralf.sommer@tuebingen.mpg.de

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PUBLISHING

The genetics of phenotypic plasticity in nematode feeding structures

Ralf J. Sommer, Mohannad Dardiry, Masa Lenuzzi, Suryesh Namdeo, Tess Renahan, Bogdan Sieriebriennikov and Michael S. Werner

Department for Integrative Evolutionary Biology, Max-Planck Institute for Developmental Biology, Spemannstrasse 37, 72076 Tübingen, Germany

RJS, 0000-0003-1503-7749

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

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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.

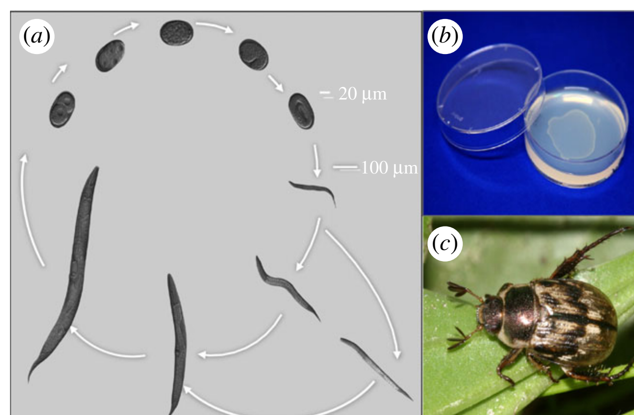

Figure 1. *Pristionchus pacificus* and growth. (*a*) Adult hermaphrodites lay eggs that develop through four larval stages to become adult. The first juvenile stage remains in the eggshell in *P. pacificus*. Under harsh and unfavourable conditions, worms develop into an arrested and long-lived dauer 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

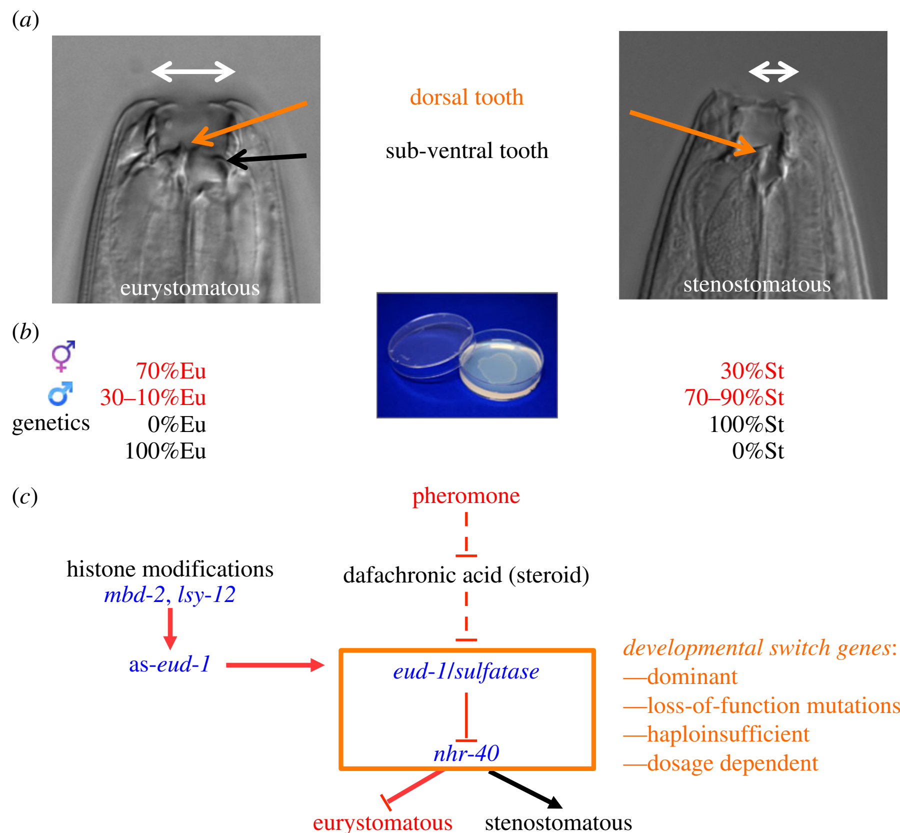

Figure 2. Genetic regulation of phenotypic plasticity of *P. pacificus* feeding structures. (*a*) Mouth dimorphism. During larval development, *P. pacificus* individuals make an irreversible decision to develop a eurystomatous morph with two teeth (orange and black arrows) and a broad buccal cavity (white 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.

Funding. The work described in this study was funded by the Max-Planck Society to R.J.S.

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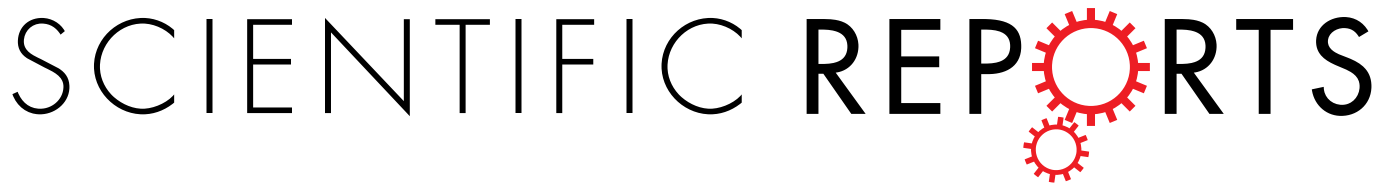

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Environmental influence on *Pristionchus pacificus* mouth form through different culture methods

Received: 8 May 2017
Accepted: 28 June 2017
Published online: 03 August 2017

Michael S. Werner, Bogdan Sieriebriennikov, Tobias Loschko, Suryesh Namdeo, Masa Lenuzzi, Mohannad Dardiry, Tess Renahan, Devansh Raj Sharma & Ralf J. Sommer

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

Department of Evolutionary Biology, Max Planck Institute for Developmental Biology, 72076, Tübingen, Germany. Correspondence and requests for materials should be addressed to R.J.S. (email: ralf.sommer@tuebingen.mpg.de)

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,

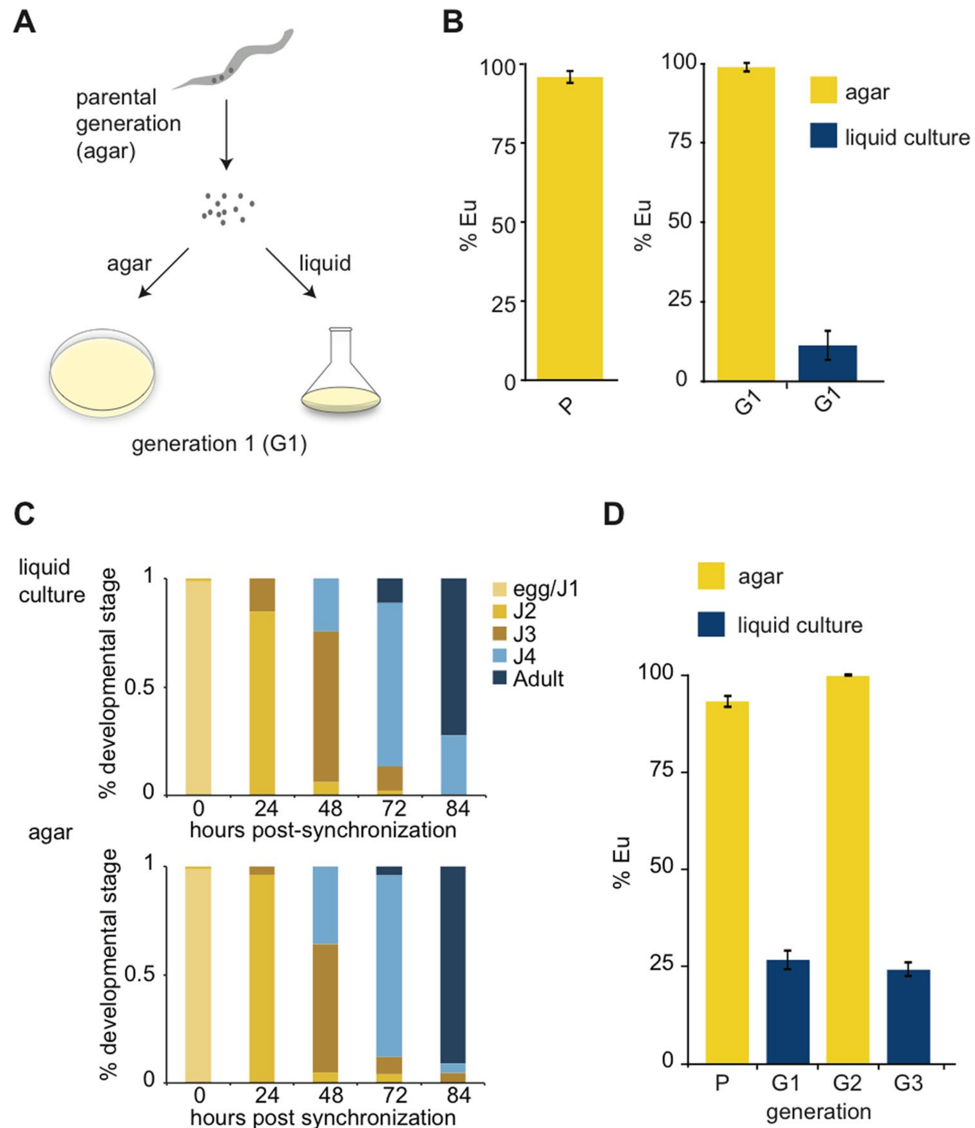

Figure 2. Different culture methods affect mouth-form phenotypic plasticity. (**A**) Diagram of experimental design to compare culture conditions from the same population after bleach synchronization. (**B**) Mouth-form ratios presented as percent eurystomatous (% Eu) from the parental generation (P) 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 ($8 \pm 4\%$). However when passed by dilution the next generation of worms exhibited intermediate Eu ratios ($51 \pm 16\%$, $p < 0.05$, *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).

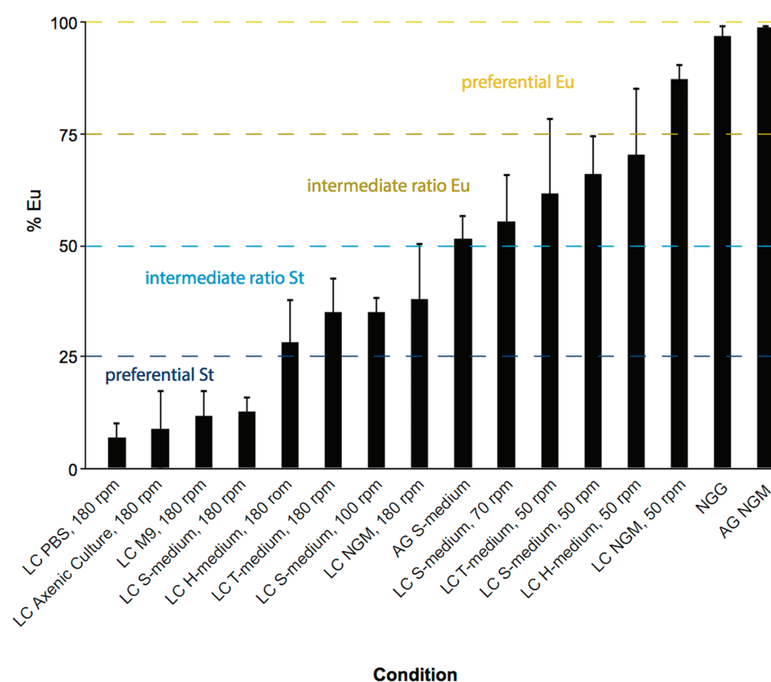

Figure 3. Comprehensive evaluation of culture method on mouth-form ratio in *P. pacificus*. Same data as in Table 1, but presented according to gradation of effect on mouth-form phenotype, from low to high % eurystomatous. LC = liquid culture, AG = agar, T and H medium = S-medium with phosphate replaced 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

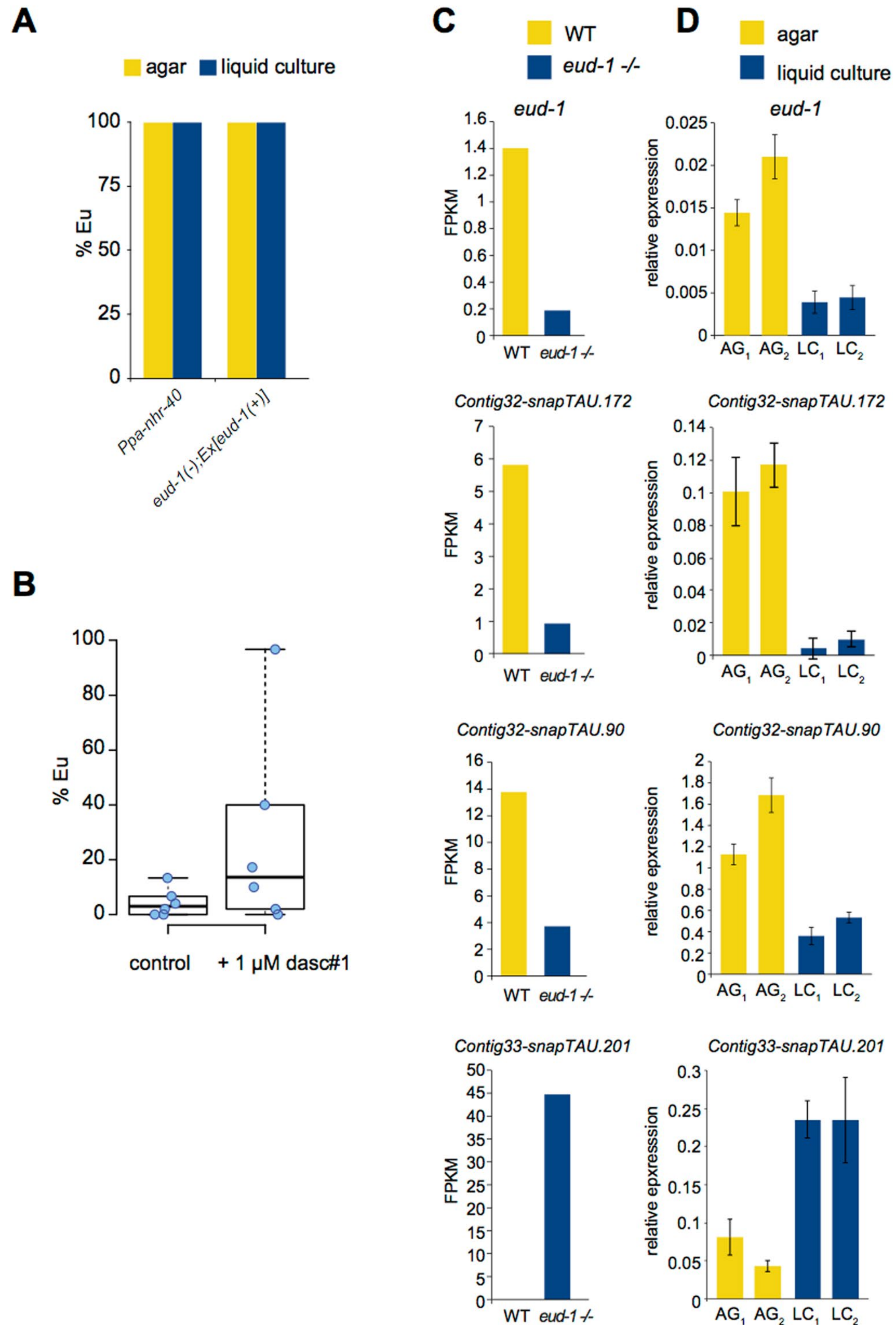

Figure 4. The environmental effect of liquid culture is upstream of known genetic components and induces similar pathways. (**A**) Mouth-form ratios of *eud-1* overexpression[27] and a *Ppa-nhr-40*[28] mutant in liquid culture reveals no effects, suggesting these genes are downstream, $n = 3$ biological 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.

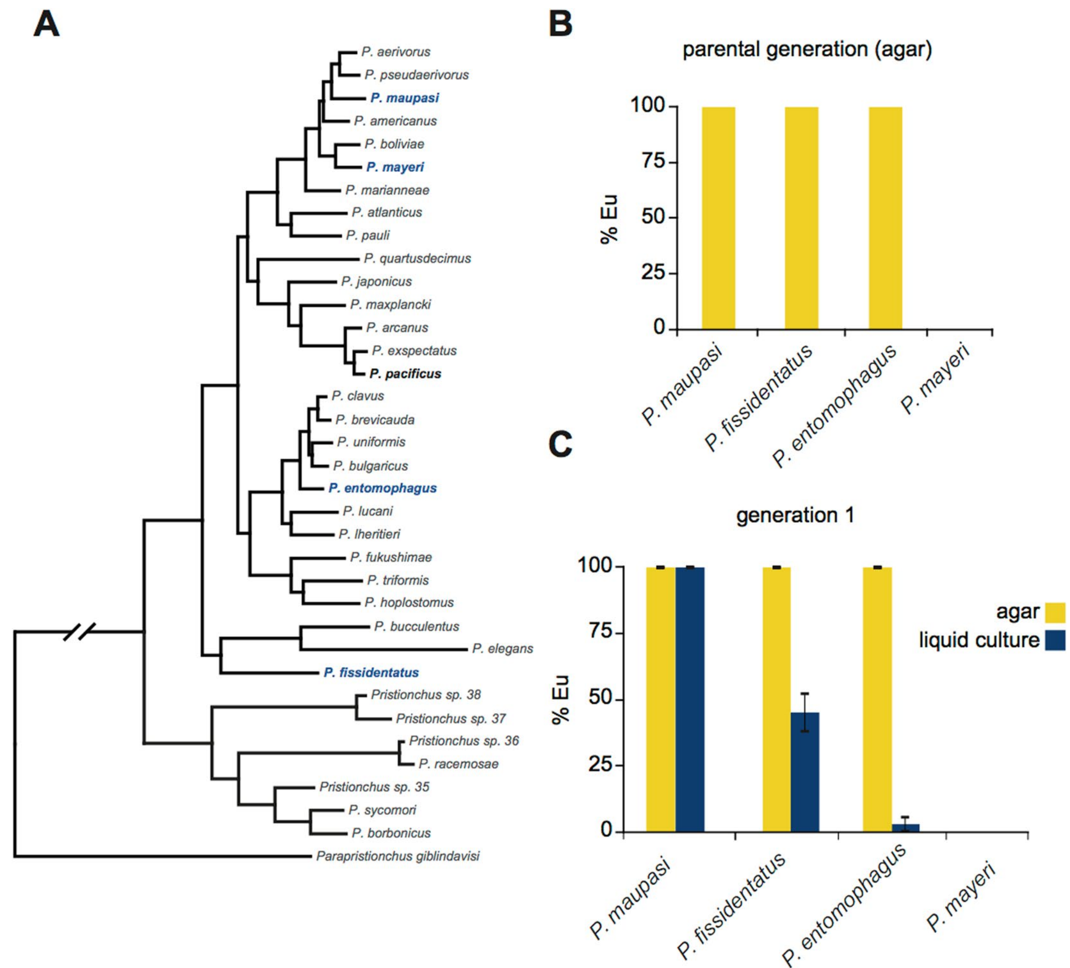

Figure 5. Macro-evolutionary view of liquid culture environmental influence. (**A**) Phylogeny of *Pristionchus* species[70] highlighting *P. pacificus* (bold), *P. fissidentatus*, *P. mayeri, and P. entomophagus* (blue). (**B**) Mouth-form ratio of parental generations ($n = 3$) of indicated species 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.

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Acknowledgements

We are thankful to current members of the Sommer laboratory for thoughtful critique of experiments, results, and interpretations, and Dr. Matthias Herrmann for assistance with the photo in Figure 1A. This study was funded by the Max Planck Society.

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

Supplementary information accompanies this paper at doi:10.1038/s41598-017-07455-7

Competing Interests: The authors declare that they have no competing interests.

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DOI: 10.1111/ede.12271

RESEARCH

WILEY EVOLUTION & DEVELOPMENT

DAF-19/RFX controls ciliogenesis and influences oxygen-induced social behaviors in *Pristionchus pacificus*

Eduardo Moreno | **Maša Lenuzzi** | **Christian Rödelsperger** | **Neel Prabh** | **Hanh Witte** | **Waltraud Roeseler** | **Metta Riebesell** | **Ralf J. Sommer**

Max Planck Institute for Developmental Biology, Department of Evolutionary Biology, Tübingen, Germany

Correspondence
Ralf J. Sommer, Max Planck Institute for Developmental Biology, Department of Evolutionary Biology, Max-Planck-Ring 9, 72076, Tübingen, Germany.
Email: ralf.sommer@tuebingen.mpg.de

Funding information
Max-Planck-Gesellschaft

Cilia are complex organelles involved in sensory perception and motility with intraflagellar transport (IFT) proteins being essential for cilia assembly and function, but little is known about cilia in an evo-devo context. For example, recent comparisons revealed conservation and divergence of IFT components in the regulation of social feeding behaviors between the nematodes *Caenorhabditis elegans* and *Pristionchus pacificus*. Here, we focus on the *P. pacificus* RFX transcription factor *daf-19*, the master regulator of ciliogenesis in *C. elegans*. Two CRISPR/Cas9-induced *Ppa-daf-19* mutants lack ciliary structures in amphid neurons and display chemosensory defects. In contrast to IFT mutants, *Ppa-daf-19* mutants do not exhibit social behavior. However, they show weak locomotive responses to shifts in oxygen concentration, suggesting partial impairment in sensing or responding to oxygen. To identify targets of *Ppa-daf-19* regulation we compared the transcriptomes of *Ppa-daf-19* and wild-type animals and performed a bioinformatic search for the X-box RFX binding-site across the genome. The regulatory network of *Ppa*-DAF-19 involves IFT genes but also many taxonomically restricted genes. We identified a conserved X-box motif as the putative binding site, which was validated for the *Ppa-dyf-1* gene. Thus, *Ppa*-DAF-19 controls ciliogenesis, influences oxygen-induced behaviors and displays a high turnover of its regulatory network.

1 | INTRODUCTION

Cilia are complex and highly conserved organelles of eukaryotic cells, involved in sensory perception, development, and motility (Pazour, Agrin, Leszyk, & Witman, 2005; Satir, Pedersen, & Christensen, 2010; Scholey, 2003). Studies in selected model organisms, such as *Caenorhabditis elegans*, *Chlamydomonas* and mice, provided detailed insight into the structure and function of cilia and ciliopathologies associated with human disease (Vincensini, Blisnick, & Bastin, 2011). In contrast, little is known about cilia development in an evo-devo context and the evolution of behaviors that require cilia function. For example, only 60 of the 302 neurons of the nematode *C. elegans* form cilia (Bae & Barr, 2008), but if and how this affects the evolution of these cells and associated processes, remain currently unknown.

The most important molecular components of cilia are intraflagellar transport (IFT) proteins organized in several sub-complexes, which are essential for proper cilia assembly and the transport of cargo molecules along the cilia axoneme, such as chemoreceptors and molecules involved in signal transduction (Taschner & Lorentzen, 2016). Based on the phenotypes of several IFT mutants, it has recently been shown

Eduardo Moreno and Maša Lenuzzi contributed equally.

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that cilia also play a role in regulating social versus solitary feeding behavior in *Caenorhabditis elegans* and *Pristionchus pacificus* nematodes (Moreno & Sommer, 2018; Moreno et al., 2017). In both *C. elegans* N2 and *P. pacificus* PS312 wild type animals solitary foraging behavior is promoted by unknown environmental inputs sensed through sensory cilia. In contrast, animals with mutations affecting IFT components display social behaviors in the form of bordering and clumping at the edge of the bacterial food lawn, where oxygen (O_2) levels are lower (Moreno & Sommer, 2018; Moreno et al., 2017). In spite of the functional conservation of most of the IFT genes analyzed, the above studies show functional divergence for some of them. For instance, *osm-1* mutants remain solitary in *C. elegans*, but become social in *P. pacificus*, while the opposite is observed in *osm-3*, coding for the homodimeric kinesin motor subunit (Moreno & Sommer, 2018; Moreno et al., 2017). These findings revealed conservation and divergence of IFT components in the regulation of clumping and bordering between both species. Additionally, it is important to note that the solitary feeding behavior of *C. elegans* N2 and *P. pacificus* PS312 wild type animals result from different evolutionary histories. In *C. elegans* and most other *Caenorhabditis* species, wild isolates are preferentially social and the solitary phenotype of *C. elegans* N2 is the result of artificial selection in the laboratory (Frézaland & Félix, 2015; McGrath et al., 2009). In contrast, in *P. pacificus* and other *Pristionchus* species most wild isolates are solitary (Moreno, McGaughran, Rödelsperger, Zimmer, & Sommer, 2016; Moreno et al., 2017). Here we complement previous comparative analyses of cilia function in nematodes by focusing on the *P. pacificus* Regulatory Factor X (RFX) transcription factor *daf-19*, the master regulator of ciliogenesis in *C. elegans* (Phirke et al., 2011; Senti & Swoboda, 2008; Swoboda, Adler, & Thomas, 2000).

RFX transcription factors control cilia formation throughout the animal kingdom (Emery, Durand, Mach, & Reith, 1996; Piasecki, Burghoorn, & Swoboda, 2010). *daf-19* is the sole RFX gene in *C. elegans*. Among the four *daf-19* isoforms that have been described in *C. elegans* only the short DAF-19c isoform regulates ciliogenesis (Swoboda et al., 2000), while the long isoforms DAF-19a-b maintain the expression levels of several synaptic proteins in all non-ciliated neurons (Senti & Swoboda, 2008) and the shortest DAF-19m isoform is required for male mating and acts specifically in the PKD and IL2 ciliated sensory neurons (Wang, Schwartz, & Barr, 2010; Wells, Rowneki, & Killian, 2015). *Cel-daf-19c* mutants lack cilia, have abnormal amphids and are defective in their ability to taste and smell (Perkins, Hedgecock, Thomson, & Culotti, 1986; Phirke et al., 2011; Senti & Swoboda, 2008; Swoboda et al., 2000). In addition, *Cel-daf-19c* mutants show constitutive dauer larva formation (*Daf-c*) phenotype (Malone & Thomas, 1994; Perkins et al., 1986). Nematode dauer larvae are arrested developmental stages adapted for surviving harsh environmental conditions, such as starvation, osmotic or temperature stress, as well as for dispersal via different host animals (Hu, 2007). *Cel-daf-19* mutants were originally identified in screens for the *Daf-c* phenotype (Malone & Thomas, 1994; Perkins et al., 1986), since they produced 70–90% dauer larvae on NGM agar plates depending on the culture temperature (Swoboda et al., 2000).

Ciliogenesis takes place during the threefold stage in *C. elegans* embryos and requires DAF-19c to promote the expression of IFT genes. The comparison of transcriptional profiling between *Cel-daf-19* mutants and wild-type animals revealed an enrichment in cilia-related genes in the set of down-regulated genes in mutants (Phirke et al., 2011). To regulate gene expression, DAF-19, like the other members of the RFX family, binds to specific DNA sequences in the promoters of its target genes called X-box motifs. The X-box motif consists of a 13–15 nucleotide imperfect palindromic sequence (Blacque et al., 2005; Efimenko et al., 2005; Swoboda et al., 2000), with one half-site being more degenerate than the other (Emery et al., 1996). In *C. elegans* previous X-box motif search projects generated several lists of putative *daf-19* target genes and allowed the identification of new IFT components (Blacque et al., 2005; Efimenko et al., 2005; Swoboda et al., 2000).

Here, we describe a broad functional analysis of the *P. pacificus daf-19* ortholog using two *Ppa-daf-19* mutants produced by CRISPR/Cas9-mediated genome engineering. First, we confirmed the conserved role of *Ppa-daf-19* in ciliogenesis by proving the absence of cilia in amphid neurons by transmission electron microscopy. Next, we analyzed behavioral and phenotypic effects of *daf-19* malfunction. Finally, we characterized the putative genetic targets of *Ppa-daf-19* searching for both conserved and newly acquired targets in embryonic transcriptome data. In addition, we performed a bioinformatic X-box motif search across the *P. pacificus* genome and validated the predicted motif for the IFT component *dyf-1*.

2 | MATERIALS AND METHODS

2.1 | Nematode strains and growth condition

The reference strain *P. pacificus* RS2333 was used in this study (a laboratory derivative of the original strain PS312 isolated from Pasadena, CA in 1988) to produce the following mutants by CRISPR/Cas9 mediated genome engineering: *daf-19(tu1035)*, *daf-19(tu1124)*, *dyf-1(tu1198)*, *dyf-1(tu1199)*, *dyf-1(tu1200)*, and *dyf-1(tu1201)*. These mutants were back-crossed with the RS2333 strain before phenotypic analysis. In addition, we employed the *P. pacificus dyf-1(tu1074)* and *ifta-1(tu1123)* mutants already available

(Moreno et al., 2017). All mutant lines and the reference strain were maintained at 20°C using standard methods (Pires da Silva, 2006).

2.2 | *Ppa-daf-19* identification and characterization

The ortholog of *daf-19* in *P. pacificus* was identified using the BLASTp algorithms implemented in http://www.wormbase.org and http://www.pristionchus.org, which corresponds to the gene prediction UMM-S245-11.51-mRNA-1 from the most recent genome assembly (Rödelsperger et al., 2017). Three isoforms were detected from previous strand-specific RNA-seq data (Rödelsperger, Menden, Serobyan, Witte, & Baskaran, 2016), which were called *Ppa-daf-19a* (ppa_stranded_DN25985_c0_g1_i1), *Ppa-daf-19b* (ppa_stranded_DN25985_c0_g1_i3) and *Ppa-daf-19c* (ppa_stranded_DN25985_c0_g1_i2) (Figure 1a). The genetic structure of the *Ppa-daf-19a* isoform was confirmed by RACE (Suppl. Figure S1), although the correspondence between the *Ppa-daf-19* isoforms and the four *Cel-daf-19* isoforms described so far (Senti & Swoboda, 2008; Wang et al., 2010; Wells et al., 2015) has not yet been established. The amino acid sequence alignment between *Cel-daf-19c* and *Ppa-daf-19c* has revealed a highly conserved RFX DNA-binding domain (DBD) and a dimerization domain (DD) (Emery, Durand, Mach, & Reith, 1996), while regions B and C were less conserved (Figures 1a and S2).

2.3 | CRISPR/Cas9 mutagenesis

Mutants were generated using the CRISPR/Cas9 system following the protocol of Witte et al. (2015). sgRNAs were synthesized by Integrated DNA Technologies Inc. (Coralville, IA). We used Cas9 protein produced by New England BioLabs Inc. (Ipswich, MA). Injection Master Mix was prepared following the manufacturer instructions from Alt-RTM CRISPR-Cas9 System User guide from Integrated DNA Technologies Inc. Injections were performed on a Zeiss Axiovert microscope (Zeiss, Germany) coupled to an Eppendorf TransferMan micromanipulator and Eppendorf FemtoJet injector (Eppendorf AG, Hamburg, Germany). Injected mothers were kept individually on NGM plates for 16 hr. Around 100 F1 progeny were isolated onto NGM plates before they became adults. Once they had laid eggs, F1 individuals were lysed and assayed for the presence of a molecular lesion around the sgRNA target site. This was performed by high resolution melting using a LightCycler 480

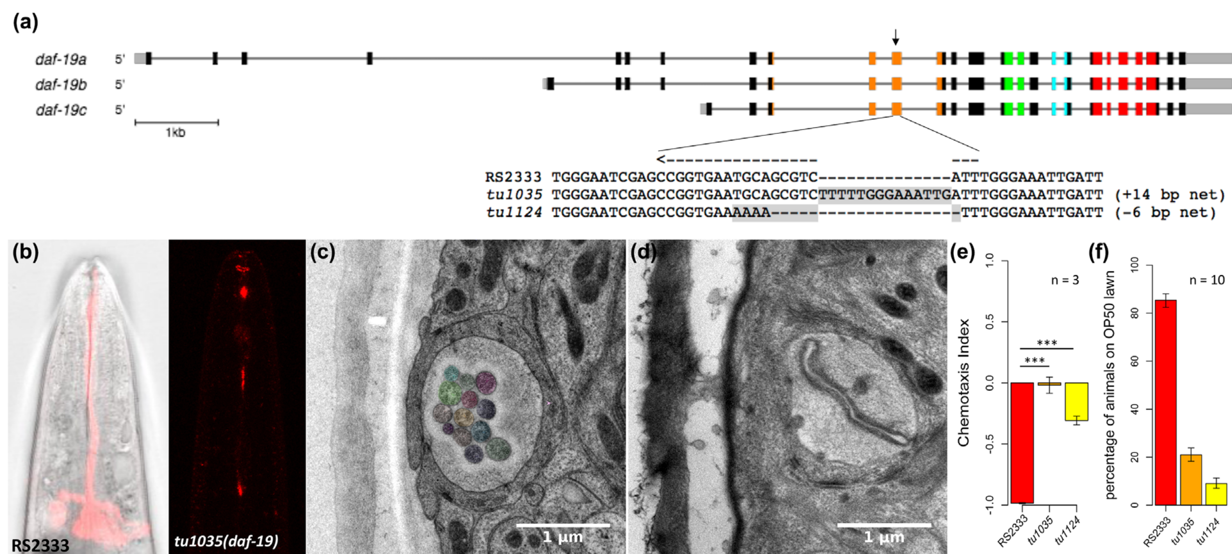

FIGURE 1 *Ppa-daf-19* gene structure and mutant phenotypes. (a) Gene structure of the *Ppa-daf-19* gene showing three isoforms identified by RNA-seq. Gray boxes indicate UTRs, black and colour boxes indicate exons with the DNA-binding domain (DBD) labeled in orange, the dimerization domain (DD) in red and regions B and C in green and blue, respectively. The arrow marks the position of the guide RNA used for CRISPR/Cas9-mediated engineering. The molecular lesions of the two *Ppa-daf-19* alleles are shown below; the arrow indicates the sgRNA sequence and the molecular modifications are highlighted in gray. (b) Overlay of bright field and fluorescent images showing dye-filling staining of amphid neurons in a RS2333 adult individual (left) and absence of dye-filling staining of amphid neurons in *Ppa-daf-19* mutants (right). (C and D) TEM micrographs of the right amphid channel in transverse sections of high-pressure-frozen and freeze-substituted adult hermaphrodite worms; dorsal side up. (c) Amphid channel of wild-type *P. pacificus* containing ciliary endings of amphid neurons labeled with false color overlays. In this section, taken about 1.6 μm from amphid opening, the channel is surrounded by the amphid socket cell. (d) The amphid channel of a *tu1035* mutant animal in a comparable position does not contain cilia and has collapsed to a slit. (e) 1-Octanol avoidance behavior. (F) Chemoattraction toward *E. coli* OP50. Bar-plot error bars in (e) and (f) represent the standard error of the mean (SEM). For statistical analysis see Suppl. Tables S2 and S3

High Resolution Melting Master (Roche Diagnostics Ltd., Burgess Hill, England). The identified mutant candidates were confirmed by Sanger sequencing. Primers for detecting gene lesions are listed in Supplementary Table S1.

2.4 | Behavioral assays

Chemotaxis assays to quantify 1-Octanol avoidance were performed following the protocol of Moreno et al. (2017). In brief, synchronized adult nematodes were loaded into 8.5-cm NGM plates between the test compound (1 µl of 100% 1-Octanol [Sigma–Aldrich Co., St. Louis, MO]) and the control compound (1 µl of 100% Ethanol [Merck Millipore, Merck KGaA, Darmstadt, Germany]), located at opposed ends of the plates. Plates were incubated at 20°C for 12 hr and then the animals within a 2 cm radius-circle around each odor source were counted. Three replicates were carried out for each strain/mutant. To assess chemoattraction toward the standard nematode food source *Escherichia coli* OP50, 50 adult nematodes were located at one end of a 6 cm NGM-agar plate containing 20 µl of *E. coli* OP50 spotted 1 Day before at the opposite end. The number of individuals that reached the OP50 lawn was scored after 8 hr of incubation at 20°C. Ten replicates were carried out for each strain/mutant. The assay for social behaviors (bordering and clumping) and O_2-induced locomotive behaviors were performed as previously established for *P. pacificus* (Moreno et al., 2016; Zimmer et al., 2009).

2.5 | Dye-filling

The dye-filling protocol to assay dye-filling defects in the amphid neurons of *Ppa-daf-19* and *Ppa-dyf-1* mutants was performed as previously described (Moreno et al., 2017). In brief, animals were stained with VybrantDiI Cell-Labelling Solution (Thermo Fisher Scientific, Waltham, MA) diluted 100-fold in 250 µl of M9 medium. After an incubation time of 3 hr on a rocking wheel, nematodes were washed three times with M9 medium. Adult individuals were picked to agar pads containing 0.3% w/v NaN3 and imaged using a Leica TCS SP8 confocal microscope. Average intensity Z-projections and overlays of bright field and fluorescent images were created using FIJI software (Schindelin et al., 2012). Intensity ranges displayed in the fluorescent images in Figures 1 and 4 are identical.

2.6 | Brood size and dauer larvae quantification

Total brood sizes of RS2333 and *Ppa-daf-19* mutant animals were quantified under standard culture conditions by picking 10 individual J4 worms per strain onto NGM-agar plates (1 worm per plate) and transferring them to new plates every 48 hr. For each individual, the numbers of J4 and dauer larvae on each plate were quantified and combined. Dauer assays under dauer-inducing environmental conditions were performed by placing 100 young adult nematodes on 6 cm NGM agar plates seeded with 300 µl *E. coli* OP50. These plates were incubated at 27°C for 12 days. To estimate the percentage of dauers, four random areas of 5 mm^2 were defined per plate and the total number of animals as well as the number of dauers inside these areas were counted under Zeiss Discovery V12 and V20 and Zeiss Stemi2000 steromicroscopes (Zeiss, Germany). Two biological replicates with three plates per replicate were performed.

2.7 | RNA-seq and transcriptome analysis

Embryos of RS2333, *Ppa-daf-19(tu1035)* and *Ppa-daf-19 (tu1124)* mutants were grown on five NGM plates each for 1 week. Embryos were isolated by hypochlorite treatment and immediately frozen in 250 µl of TRIzol® Reagent solution (Ambion, Life Technologies, Carlsbad, CA) and stored at −80°C prior to being used for total RNA extraction. Total RNA was extracted with the ZymoBIOMICS DNA/RNA Miniprep Kit (ZymoBIOMICS®, Irvine, CA). Transcriptome libraries were produced with the TruSeq RNA Library Prep Kit v2 from Illumina (Illumina Inc., San Diego, CA). DNA was sheared to 350 bp using the Covaris S2 System (Covaris Ltd., Woodingdean Brighton, United Kingdom) and end repair, adenylation, adaptor ligation and PCR amplification were performed following the kit protocol. Libraries were validated on an Agilent Bioanalyzer DNA 1000 chip (Agilent Technologies GmbH, Waldbronn, Germany) and pooled before sequencing on an Illumina HiSeq. 3000 platform. RNA-seq reads were aligned against the *P. pacificus* Hybrid1 genome with the help of the tophat2 aligner (version 2.1.1, default options). Differentially expressed genes were identified by the program cuffdiff (version 2.2.1) from the comparison of both *Ppa-daf-19* alleles with two wildtype control samples. The sets of up- and down-regulated genes (FDR corrected *p*-value <0.05) in the two *Ppa-daf-19* alleles were intersected to define 217 commonly up-regulated and 214 commonly down-regulated genes (Suppl. Data File 1).

2.8 | Transmission electron microscopy

Two sets of serial TEM sections covering the anterior 10–15 µm of two adult hermaphrodites of *P. pacificus tu1035* were prepared as follows: Worms were high-pressure frozen with a Bal-tec HPM-10 high-pressure freezer, processed through freeze substitution using a Leica EM AFS2 freeze substitution unit and embedded in Epon. Substitution and embedding were carried out according to Bumbarger, Riebesell, Rödelsperger, and Sommer (2013). Blocks were

sectioned with a Reichert Ultracut E microtome at 50–60 nm. Sections were picked up on slot grids, dried in an oven at 60°C, post stained with 2% uranyl acetate in 70% methanol and 0.4% lead citrate. The anterior-most 200 to 300 sections of the worms were imaged with a TVIPS TemCam F416 4 K camera mounted on a Tecnai G2 spirit transmission electron microscope. The resulting images were montaged, aligned and manually segmented using TrakEM2 (Cardona et al., 2012; Kremer, Mastronarde, & McIntosh, 1996). As wild type reference we used two existing serial TEM section sets of adult hermaphrodites of *P. pacificus* strain PS312 (Bumbarger et al., 2013). For a detailed method description see also protocol 9 of the *P. pacificus* protocols in WormBook (Pires-daSilva, 2013).

2.9 | Motif analysis

In order to define a candidate gene set of direct DAF-19 targets, we first searched the X-box consensus GTTNCCATGGNAAC from Swoboda et al. (2000), GTYNCYATRGYAAC from Blacque et al. (2005), and GTHNYYATRRNAAC from Efimenko et al. (2005) in the *P. pacificus* Hybrid1 assembly. As these searches only yielded 10, 48, and 638 hits in the complete genome, we screened for homologous transcription factors with better characterized binding profiles in the UniPROBE data bases (Hume, Barrera, Gisselbrecht, & Bulyk, 2015) of in vitro determined binding affinities. Protein sequence similarity searches with BLASTP yielded the mouse DNA-binding domains of mouse proteins Rfx3, Rfx4, and Rfxdc2 as homologs of *Ppa-daf-19*, all of which bind the consensus sequence GTTGCYANG. The search for this motif in the *P. pacificus* genome identified 4,827 putative DAF-19 binding sites.

2.10 | Statistical analyses

Statistical analyses were performed in the computing environment R ver. 3.4.2 (R CoreTeam, 2017). Replicates of aerotaxis and chemotaxis, as well as brood size quantification assays were used to calculate means and standard errors (S.E.M.). Two-sample equal variance Student's t-test, with Bonferroni corrections for multiple hypothesis testing, was used to confirm significant differences in averages between wildtype and mutant strains. For analysis of dauer, bordering and clumping phenotypes, recorded as proportional data, we performed logistic regression using a binomial error structure and a logit link function. With this analysis, we specifically tested for differences among *Ppa-daf-19* and *Ppa-dyf-1* mutant lines. Replicates within lines were included in the model, and significance was assessed using a z-test. Pairwise comparisons between lines and biological replicates were performed by the Least-Squares Means method from the R package lsmeans and significance thresholds were set using the false discovery rate correction method.

3 | RESULTS

3.1 | *Ppa-daf-19* mutants lack cilia in amphid neurons

To analyze the morphological and behavioral effects of mutations in the *Ppa-daf-19* locus, we applied the CRISPR/Cas9 technology on the wild-type strain RS2333 using a guide RNA designed to target the DNA binding domain that is present in all three alternatively spliced isoforms of *Ppa-daf-19* (Figure 1a). We obtained two mutant lines, one frame shift mutant (*tu1035*) inducing a premature stop codon and one in-frame mutant (*tu1124*) with a deletion of 12 bp that result in the absence of four amino acids (NAAS) at the beginning of the third alpha helix (Gajiwala et al., 2000) and an insertion of two lysines instead (Figures 1a and S3). While the two alanines are part of the side chains buried on DNA binding (Gajiwala et al., 2000), any of the four amino acids deleted in *tu1124* make direct or water-mediated DNA contact (Piasecki et al., 2010). Therefore, both mutants most likely represent *reduction-of-function* alleles.

First, we assayed dye-filling defects in the amphid neurons of *Ppa-daf-19* mutants following the dye-filling protocol previously stablished for *P. pacificus* (Moreno et al., 2017, see Section 2). Both *Ppa-daf-19* mutant lines were dye-filling defective (Figure 1b) indicating morphologic defects in ciliated neurons. To characterize the morphology of the cilia in the *Ppa-daf-19* mutants we examined ultra-thin sections of the tip of the head region of two *tu1035* specimens using transmission electron microscopy (TEM). Two complete wild-type TEM serial section data sets prepared previously (Bumbarger et al., 2013) served a reference (Figure 1c). In each of the two mutant worms one of the amphid structures was clearly discernible showing sheath cell and socket cell forming an amphid channel with a pore to the outside, but the channel does not contain cilia, the lumen is empty and has collapsed to a slit (Figure 1d). One of the amphid sheath cells could be followed for about 14 μm to the posterior end of the series without observing a neuronal process entering it. In the wild-type series, sheath entry starts at about 12 μm from the tip of the worm, that is, within in the range that is covered by the *Ppa-daf-19* series. Thus, the amphid neurons of the *Ppa-daf-19* mutants lack cilia in the channel and the dendrites do not enter the sheath cells in the position they do in wild-type. Unfortunately, oblique orientation and gaps of unknown width in both *Ppa-daf-19* series were obstacles to a more thorough analysis and several of the dendrites and glia cells of the head sensory neurons were impossible to identify.

For the labial and cephalic neurons, the damage seems to be less severe than in the amphid neurons with regard to

sheath entry and remnants of ciliary structures, but regular ciliated endings were not found. In the more complete series, we found all four CEP dendrites (Suppl. Figure S4), four of the IL1 dendrites and one of the OL dendrites to enter their sheath cells and end there after 0.4 to 0.6 μm. Only in two cases, one in CEP, one in IL1, dendrites were found to reach the socket cell and end in the cuticle. Apart from rootlet-like structures in two CEP dendrites and some free microtubules in one CEP and one IL1, no ciliary structures were detected, that is, no doublet microtubules, no transition zone, middle or distal segments (Suppl. Figure S4).

3.2 | *Ppa-daf-19* mutants show chemosensation defects

Since the amphid constitute the primary chemosensory organ of nematodes (Bargmann, 2006), the absence of cilia in amphid neurons of *Ppa-daf-19* mutants should be reflected in chemosensory-defective phenotypes. To test this hypothesis, we first analyzed the response of *Ppa-daf-19* mutants to 1-Octanol, a chemical compound that induces strong avoidance in *P. pacificus* wild type animals (Cinkornpumin et al., 2014). We found that *Ppa-daf-19* mutants failed to avoid 1-Octanol (Figures 1e and Table S2). In addition, we tested the ability of *Ppa-daf-19* mutants to detect the bacterial food source *E. coli* OP50. While more than 80% of wild-type animals were able to reach the OP50 lawn within 8 hr, only 10–20% of the mutant individuals were able to do so (Figures 1f and Table S3). These findings indicate that *Ppa-daf-19* mutants are either not able to smell the presence of bacteria or are not attracted to it. It should be noted, however, that most of the individuals that reached the bacterial lawns remained on them.

3.3 | *Ppa-daf-19* mutants have no dauer phenotype but reduced brood size

daf-19 mutants in *C. elegans* are dauer-formation constitutive, *Daf-c* (Swoboda et al., 2000). In contrast, we did not observe a *Daf-c* phenotype in *Ppa-daf-19* mutant animals. Specifically, none of the two *Ppa-daf-19* mutants produced any dauer larvae at 20°C on standard NGM agar plates (Figure 2a & Table S4). Therefore, we tested whether the absence of dauer larvae in *Ppa-daf-19* mutants was due to a dauer formation defective (*Daf-d*) phenotype. For this we exposed nematodes to dauer-inducing environmental conditions (starvation plus temperature stress, see methods). In two replicates, 20–50% of wild-type animals formed dauer larvae, while only 10–20% of *Ppa-daf-19* mutants entered the dauer stage (Figures 2b & Table S5). This finding indicates that *Ppa-daf-19* mutants are neither completely *Daf-d* nor *Daf-c*. During the quantification of dauer larvae at 20°C, we observed that egg-laying and the development of the brood size over time was delayed in *Ppa-daf-19* mutants (Figure 2c). Also, we observed a significant reduction in the brood size of *Ppa-daf-19* mutants compared to wild type (Figures 2d and Table S6). Thus, the fecundity of *Ppa-daf-19* animals is lower than that of wild type animals.

3.4 | *Ppa-daf-19* mutants show impaired behaviors related to oxygen sensing

To further investigate the relation between cilia and social behavior, we studied *Ppa-daf-19* mutants using assays for bordering and clumping behaviors that were previously established for *P. pacificus* (Moreno et al., 2016). In contrast to IFT mutants (Moreno et al., 2017), *Ppa-daf-19* mutants do

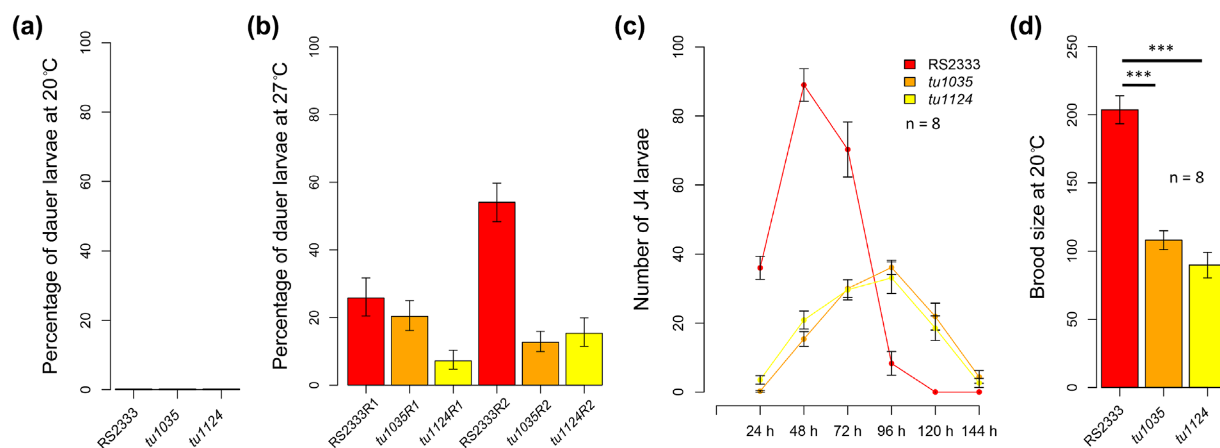

FIGURE 2 Brood size and dauer phenotypes of RS2333 and *Ppa-daf-19* mutants. (a) Percentage of dauer larvae under standard culture conditions at 20°C. (b) Percentage of dauer larvae after 12 days incubation at 27°C. The experiment was replicated twice. For graphical representation, dauer larvae frequencies were pooled across technical replicates within lines and biological replicates, with confidence intervals estimated using a binomial test. (c) Distribution of brood size over time under standard culture conditions at 20°C. (d) Total brood size under standard culture conditions at 20°C. In all graphs bar-plot error bars represent the SEM. For statistical analysis see Suppl. Tables S4 and S6

not exhibit bordering and clumping, but spread evenly in a lawn of OP50 instead, similarly to wild type animals (Figure 3a and Tables S7-8).

To test whether the solitary behavior of *Ppa-daf-19* mutants was due to defects in oxygen sensation we analyzed O_2-induced changes in locomotion speed and turning behavior produced by shifts in O_2 concentration from 21% to 16% and back to 21% (Moreno et al., 2016; Zimmer et al., 2009). Wild-type nematodes responded strongly to the shift from 16% to 21% by increasing the rate of omega (Ω) turns, whereas the two *Ppa-daf-19* mutants showed a much weaker Ω-turn response (Figure 3b and Table S9). In addition, wild-type nematodes modulated the locomotive speed according to the [O_2], which was not the case for the *Ppa-daf-19* mutants (Figure 3c). Note that the foraging speed of *Ppa-daf-19* mutants was only half of that of the wild-type animals at 21% O_2 concentration (Figure 3c and Table S10). Taken together, these experiments reveal that *Ppa-daf-19* mutants may show impaired O_2 sensing, or alternatively, may be physically unable to respond to O_2 shifts as well as wild-type animals, or both.

3.5 | IFT-related genes are down-regulated in *Ppa-daf-19* mutant embryos

To characterize the regulatory network of cilia formation in *P. pacificus,* we compared the embryonic transcriptomes of the two *Ppa-daf-19* mutants with those of wild-type animals. We found that cilia-related genes show a trend towards down-regulation in mutants. Specifically, we identified 214 (less than 1%) of the 30,884 predicted genes to be down-regulated and 317 (1%) genes that were up-regulated in both alleles (Suppl. Data File 1). The proportion of gene homology classes among differentially expressed genes roughly reflects their overall genome-wide distribution (Baskaran et al., 2015). Specifically, 56 (24%) down-regulated genes and 60 (19%) up-regulated genes have a 1:1 orthologue in *C. elegans* (Suppl. Data File 1). Similarly, 53 (25%) down-regulated and 50 (16%) up-regulated genes were defined as orphan genes (Baskaran et al., 2015, Suppl. Data File 1) and could represent putative newly acquired targets of *daf-19*.

Interestingly, none of the up-regulated genes was found to have known functions in cilia formation, whereas 27 of the down-regulated genes have known function in cilia structure and function. This includes IFT components, modulators of IFT, as well as genes expressed in cilia, such as chemoreceptors (Suppl. Data File 1). These results constitute a significant enrichment in cilia-related genes ($p < 10^{-15}$, Fisher's exact-test). Thus, the *Ppa-daf-19* transcriptomes reveal an overall trend towards down-regulation of cilia-related genes similar to previous studies in *C. elegans* (Phirke et al., 2011), but at the same time the *Ppa*-DAF-19 regulatory network involves a certain number of novel genes.

3.6 | The *pacificus* X-box motif shows a highly conserved half site

Finally, we analyzed the DNA binding specificity of *Ppa*-DAF-19. While previous definitions of the X-box motif in *C. elegans* were only based on a handful of experimentally validated binding sites (Efimenko et al., 2005), we exploited large-scale in vitro determined binding profiles as available in the UniProbe database (Hume et al., 2015). We found that the DBD domain of *C. elegans* and *P. pacificus* DAF-19 proteins mostly resemble the DBD of other mammalian RFX transcription factors, which are reported to bind a GTTGCYANG sequence. This motif represents the more conserved half site of the X-box motif described for

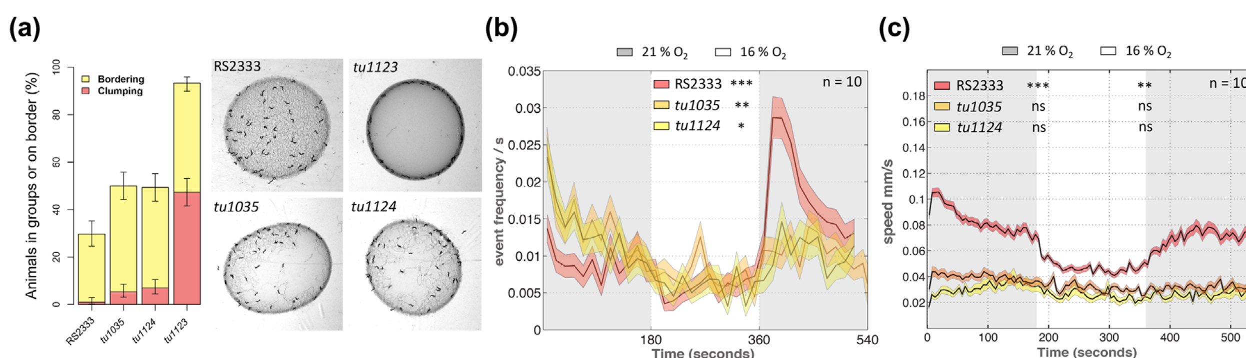

FIGURE 3 Social and O_2-induced locomotive responses of RS2333 and *Ppa-daf-19* mutants. (a) Bordering and clumping behaviors. The social *ifta-1(tu1123)* allele has been included for comparison. For graphical representation, bordering and clumping frequencies were pooled across technical replicates within lines, with confidence intervals estimated using a binomial test. For statistical analysis see Suppl. Table S7 and S8. (b) Ω-turn rate in response to 21% –> 16% –> 21% [O_2] shifts on a lawn of *E. coli* OP50 calculated in 15 s bins. (c) Locomotive speed in response to 21% –> 16% –> 21% [O_2] shifts on a lawn of *E. coli* OP50 calculated in 5 s bins. In (b) and (c) graphs, the central line represents the mean and the colored area, the SEM. For statistical analysis see Suppl. Tables S9 and S10

C. elegans (Figure 4a) (Blacque et al., 2005; Efimenko et al., 2005; Swoboda et al., 2000). Using this short motif as consensus sequence we searched the genome of *P. pacificus*, which led to the identification of 4,827 potential binding sites and translates into 567 (2%) genes with a predicted binding site in their promoter (500 bp upstream region of the translation start site). Thirteen (6%) of the 214 down-regulated genes contained a predicted binding site in their promoter, which represents a significant overrepresentation ($p < 0.001$, Fisher's exact-test). However, only 8 (3%) of 317 up-regulated genes had a predicted promoter binding site ($p = 0.3$), suggesting that *Ppa-daf-19* primarily acts as activator and that up-regulation upon *Ppa-daf-19* knock out might be a downstream effect.

To validate these X-box motif predictions, we targeted the predicted single X-box motif of the *Ppa-dyf-1* gene by CRISPR/Cas9 engineering. *dyf-1* encodes for one of the IFT components, which is required to dock the OSM-3 kinesin onto the IFT complex (Ou, Blacque, Snow, Leroux, & Scholey, 2005). We obtained four independent mutants, which lack either the complete X-box motif or its upstream half (Figure 4a). Strikingly, all four mutants show the same phenotypes as the previously described *Ppa-dyf-1* mutants (*tu1072* and *tu1074*), which bear mutations in the coding regions (Moreno et al., 2017). Specifically, these mutants are dye-filling defective (Figure 4b), fail to avoid 1-Octanol (Figure 4c and Table S11) and display strong bordering and clumping behaviors (Figure 4d and Tables S12–13). Taken

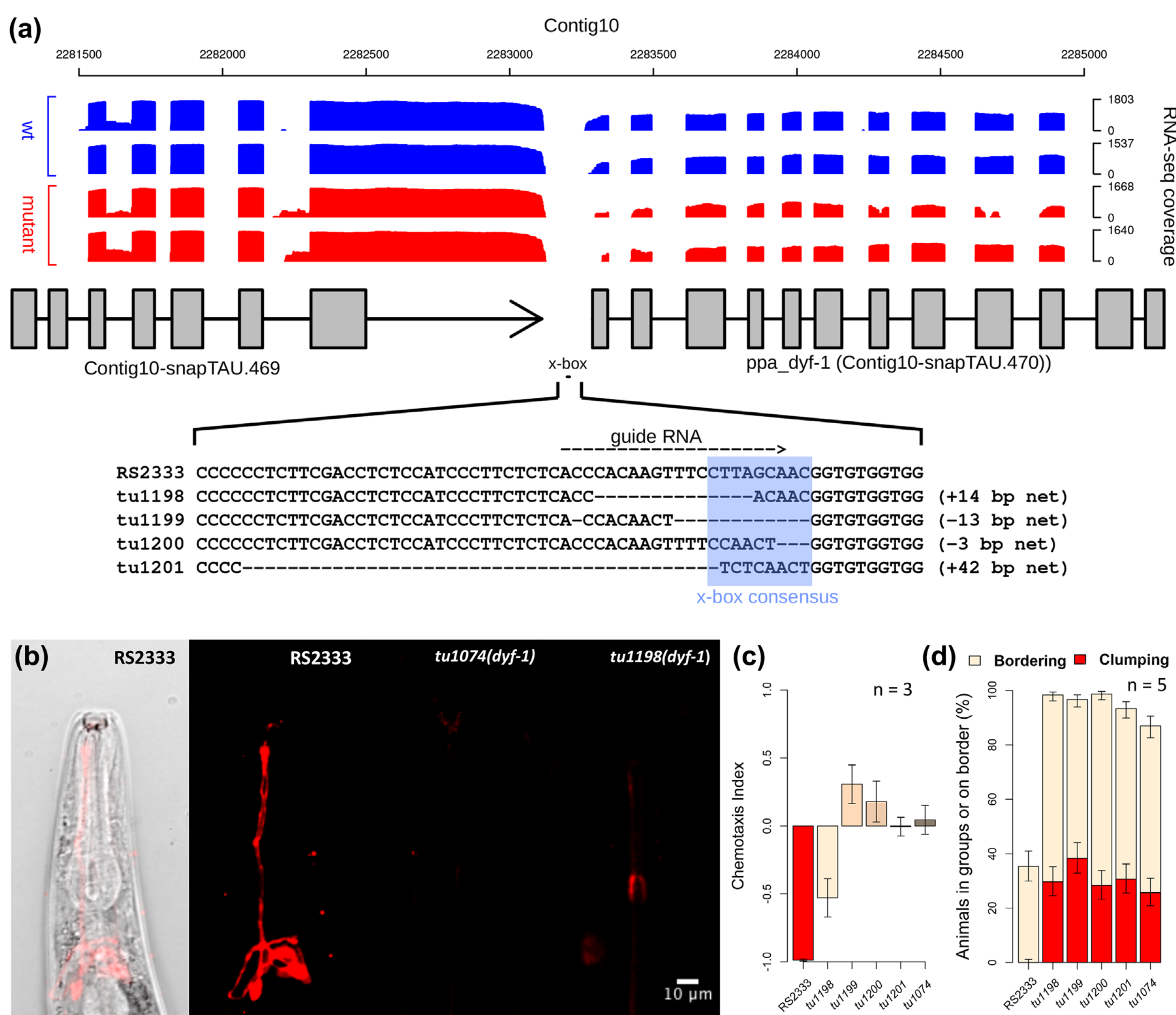

FIGURE 4 Phenotypes of the X-box *Ppa-dyf-1* mutants generated in this study. (a) Induced genetic lesions of the four mutant alleles produced in the X-box motif of the *Ppa-dyf-1* gene. The blue shading represents the X-box consensus sequence in the reverse DNA strand. (b) Overlay of bright field and fluorescent images showing dye-filling staining of amphid neurons in a RS2333 adult individual (left) and dye-filling staining of amphid neurons in RS2333 and two *Ppa-dyf-1* mutants. (c) 1-Octanol avoidance behavior. Bar-plot error bars represent the SEM (d) Bordering and clumping behaviors. For graphical representation, bordering and clumping frequencies were pooled across technical replicates within lines, with confidence intervals estimated using a binomial test. For statistical analysis see Suppl. Tables S11 and S12

together, our phenotypic analysis of *Ppa-daf-19* and *Ppa-dyf-1* promoter and coding region mutants (Moreno et al., 2017) in combination with our bioinformatic analysis would be consistent with the upstream half of the X-box motif being necessary for binding to DAF-19/RFX in *P. pacificus*. This most parsimonious hypothesis is in large parts due to similarities with other systems and awaits final proof through biochemical investigations.

4 | DISCUSSION

Here we describe the isolation and characterization of two mutants in the *Ppa-daf-19* gene, encoding the single RFX transcription factor in nematodes. Functional characterization extends previous studies on IFT mutants with respect to the regulation of social behavior and oxygen sensing. Together, our studies result in three major conclusions.

First, we observed strong differences in *daf-19* function between *P. pacificus* and *C. elegans* during dauer induction. One of the most striking features found in *Cel-daf-19* mutants is the *Daf-c* phenotype, which is exclusively associated with the DAF-19c isoform (De Stasio et al., 2018; Senti & Swoboda, 2008). Our analysis has revealed that none of the *Ppa-daf-19* mutants show a *Daf-c* phenotype, indicating divergence in the dauer regulatory pathway between the two species. While the transcription factors *daf-12*/NHR and *daf-16*/Forkhead show *Daf-d* phenotypes in *P. pacificus* similar to *C. elegans* (Ogawa, Bento, Bartelmes, Dieterich, & Sommer, 2011; Ogawa, Streit, Antebi, & Sommer, 2009), other studies have shown functional divergence from *C. elegans* dauer regulating genes. For example, a previous study found that *daf-21*, a member of the Hsp90 family of molecular chaperones has a *Daf-c* phenotype in *C. elegans*, but not in *P. pacificus* (Sieriebriennikov, Markov, Witte, & Sommer, 2017). Thus, our findings provide another example for orthologous genes with divergent functions in the regulation of dauer entry between nematode species. On a more general level, this further supports the assumption of developmental systems drift as previously observed for the regulation of vulva development between *P. pacificus* and *C. elegans* (Wang & Sommer, 2011).

Second, while IFT mutants display social feeding behavior in both *C. elegans* and *P. pacificus*, *Ppa-daf-19* mutants remain solitary. However, our additional experiments suggest that *Ppa-daf-19* mutants are unable either to sense or to respond properly to the hyperoxic conditions that normally promote social behavior. Specifically, the analysis of O_2-induced locomotive behaviors revealed a weak omega-turn response to increased [O_2] shifts, whereas there was no response in locomotion. In *C. elegans,* O_2 levels are monitored by means of ciliated and non-ciliated neurons (Coates & de Bono, 2002; Zimmer et al., 2009). In *P. pacificus* and *C. elegans*, IFT mutants are able to sense high O_2 levels and perform O_2-induced behaviors in spite of defective cilia. In the case of *C. elegans* IFT mutants, the non-ciliated URX sensory neuron may be completely functional and trigger the responses to O_2. Thus, the O_2-induced phenotypes observed in *Ppa-daf-19* mutants suggests that the knockout of the *daf-19* locus also affects the function of non-ciliated neurons involved in O_2 sensing, which results in a constant solitary foraging behavior despite shifts in O_2 concentration. Alternative, these results may indicate that either the neuron responsible for O_2 sensing in *P. pacificus* is ciliated but is not the URX cellular homolog, or that the URX cellular homolog is ciliated in *P. pacificus*. Future studies will be needed to determine whether the URX neuron is ciliated in *P. pacificus* and its role in O_2 sensation.

Finally, the analysis of the embryonic transcriptomes of *Ppa-daf-19* mutants and wild-type animals revealed a significant enrichment in cilia-related genes ($p < 10^{-15}$, Fisher's exact-test), further supporting the role of *daf-19* as the main transcription factor regulating ciliogenesis in *P. pacificus.* In addition, we identified an X-box motif with a well-conserved upstream half site as the putative binding site for *Ppa-daf-19*. It is present in the promoter regions of nine of the 11 *P. pacificus* IFT genes, which are down-regulated in both *Ppa-daf-19* mutants. The finding that the knockout of the X-box motif in the promoter region of the IFT gene *Ppa-dyf-1* results in the same phenotype as those mutants previously found in coding regions, indicates that the predicted X-box motif corresponds to the actual binding site necessary for transcription of *Ppa-dyf-1*. Together, these experiments suggest that ciliogenesis in *P. pacificus* is controlled by X-box dependent regulation of target genes by DAF-19. At the same time, our results show that a certain number of target genes are non-conserved orphan or novel genes (25% of total down-regulated). Thus, the conservation of DAF-19 function at the level of its binding specificity is coupled to extensive rewiring of the *daf-19* regulatory network resulting in different organismal phenotypes of *Cel-daf-19* and *Ppa-daf-19* mutants.

ACKNOWLEDGMENTS

We thank Drs M. Werner and J. Lightfoot for reading the manuscript and for discussions. We thank the Max Planck Society for funding the research.

ORCID

Eduardo Moreno http://orcid.org/0000-0001-7536-4122

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SUPPORTING INFORMATION

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How to cite this article: Moreno E, Lenuzzi M, Rödelsperger C, et al. DAF-19/RFX controls ciliogenesis and influences oxygen-induced social behaviors in *Pristionchus pacificus*. *Evolution & Development*. 2018;20:233–243. https://doi.org/10.1111/ede.12271

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Research

Cite this article: Moreno E, Lightfoot JW, Lenuzzi M, Sommer RJ. 2019 Cilia drive developmental plasticity and are essential for efficient prey detection in predatory nematodes. *Proc. R. Soc. B* **286**: 20191089. http://dx.doi.org/10.1098/rspb.2019.1089

Received: 13 May 2019
Accepted: 16 September 2019

Subject Category:
Behaviour

Subject Areas:
behaviour, neuroscience

Keywords:
Pristionchus pacificus, cilia, mouth-form plasticity, predatory behaviour, self-recognition

Author for correspondence:
Ralf J. Sommer
e-mail: ralf.sommer@tuebingen.mpg.de

Electronic supplementary material is available online at http://dx.doi.org/10.6084/m9.figshare.c.4674251.

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Cilia drive developmental plasticity and are essential for efficient prey detection in predatory nematodes

Eduardo Moreno, James W. Lightfoot, Maša Lenuzzi and Ralf J. Sommer

Department of Evolutionary Biology, Max Planck Institute for Developmental Biology, Max-Planck-Ring 9, 72076 Tübingen, Germany

RJS, 0000-0003-1503-7749

Cilia are complex organelles involved in a broad array of functions in eukaryotic organisms. Nematodes employ cilia for environmental sensing, which shapes developmental decisions and influences morphologically plastic traits and adaptive behaviours. Here, we assess the role of cilia in the nematode *Pristionchus pacificus*, and determine their importance in regulating the developmentally plastic mouth-form decision in addition to predatory feeding and self-recognition behaviours, all of which are not present in *Caenorhabditis elegans*. An analysis of a multitude of cilia-related mutants including representatives of the six protein subcomplexes required in intraflagellar transport (IFT) plus the regulatory factor X transcription factor *daf-19* revealed that cilia are essential for processing the external cues influencing the mouth-form decision and for the efficient detection of prey. Surprisingly, we observed that loss-of-function mutations in the different IFT components resulted in contrasting mouth-form phenotypes and different degrees of predation deficiencies. This observation supports the idea that perturbing different IFT subcomplexes has different effects on signalling downstream of the cilium. Finally, self-recognition was maintained in the cilia deficient mutants tested, indicating that the mechanisms triggering self-recognition in *P. pacificus* may not require the presence of fully functional cilia.

1. Introduction

Cilia are complex hair-like organelles present on most eukaryotic cells, with many diverse functions [1], and are well conserved across evolution [2]. The broad functions of cilia include roles in motility, sensing and responding to environmental cues [3,4]. Additionally, in mammals, cilia are signal transduction centres with an essential role in Hh signalling, and thus cilia are crucial for development, cell differentiation and tissue homeostasis [5,6]. Cilia consist of a microtubule-based cytoskeleton called the axoneme surrounded by cell membrane [5]. The growth and maintenance of cilia is achieved by intraflagellar transport (IFT) [7], an evolutionarily conserved motility process involving six protein subcomplexes (see electronic supplementary material, figure S1): the IFT-subcomplex A and B, the BBSome, the homodimeric and heterodimeric kinesin motors, and the dynein motor [8–10]. In humans, genetic mutations resulting in abnormal cilia formation or function give rise to a constellation of disorders named ciliopathies (such as Bardet–Biedl syndrome), which include cerebral anomalies, renal disease, retinal degeneration, anosmia, deafness, diabetes, obesity, polydactyly and skeletal dysplasias among others [11,12].

Much of our knowledge of the functions of cilia comes from studies performed in the green alga *Chlamydomonas* [13] and in the nematode *Caenorhabditis elegans* [14]. In the latter, only sensory neurons are ciliated and importantly, worms with abolished cilia are viable, which makes it a suitable model organism to study the genetic regulation of cilia development and function [15]. *Caenorhabditis elegans* possess 302 neurons, of which 60 have cilia at the ends of their dendritic processes [16]. All these ciliated neurons are sensory neurons, including the amphid,

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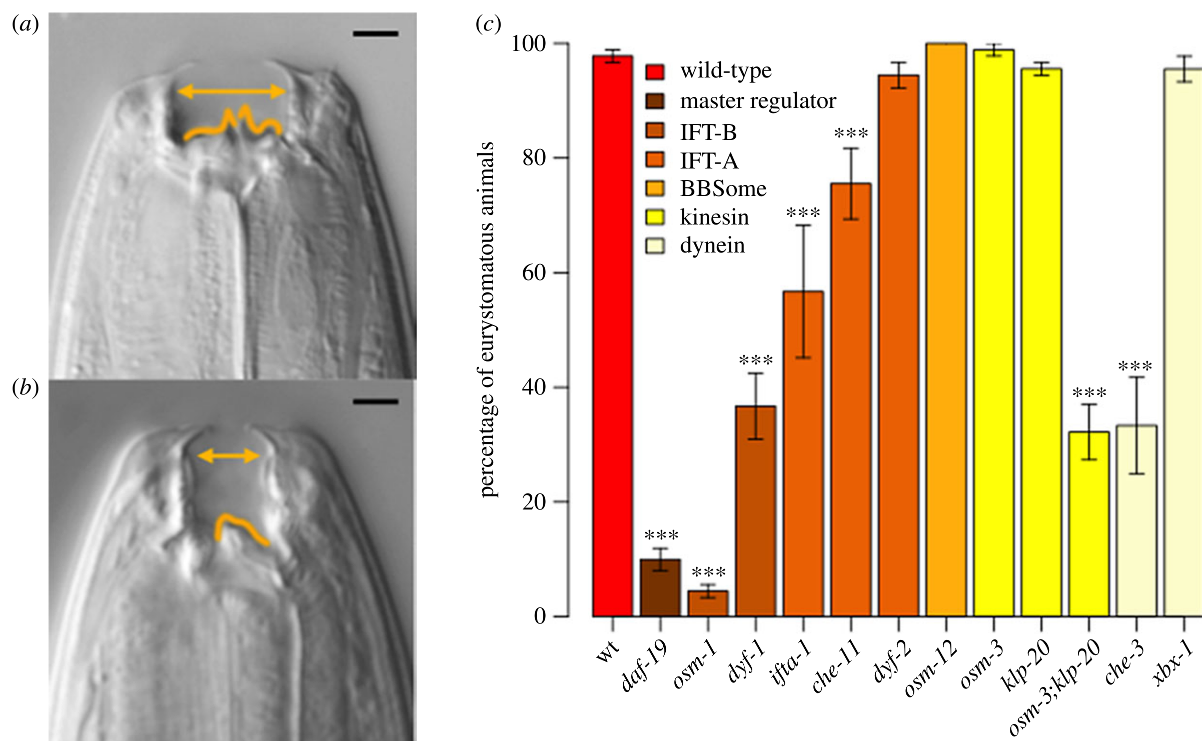

Figure 1. (*a*) Representative image of the eurystomatous morph of *P. pacificus*, characterized by a wide buccal cavity with two teeth. (*b*) Representative image of stenostomatous morph of *P. pacificus*, characterized by a narrow buccal cavity with a single dorsal tooth. Scale bars, 5 µm. (*c*) Mouth-form ratios presented as percentage eurystomatous for hermaphrodites of wild-type, *daf-19* and IFT mutants in standard NGM agar cultures. For the IFT mutants, colours indicate the IFT subcomplex to which the IFT genes belong (see also electronic supplementary material, figure S1). In all experiments, three biological replicates were performed for each line, and 30 young adult individuals were examined per replicate. Error bars represent the standard error of the mean. $*p < 0.05$; $***p < 0.001$; all the comparisons are to wild-type and only statistically significant comparisons are shown (electronic supplementary material, table S4A). (Online version in colour.)

phasmid, labial, cephalic, pseudocoelomic and deirid neurons, among others [17]. These sensory neurons are involved in a wide variety of tasks related to sensing and responding to the environment, such as detection of food sources, finding mate partners, escaping from danger or noxious conditions, and the regulation of developmental transitions such as dauer entry and recovery [18]. Mutations in *C. elegans* IFT components produce an array of phenotypes related with chemo-, osmo-, thermo- and mechanosensation, mating, dauer larvae development and dye-filling of certain amphid neurons [17,18]. Recently, we have shown that IFT components and cilia are also important to regulate hyperoxia avoidance behaviours in *C. elegans* and *Pristionchus pacificus* nematodes [19–21].

Contrary to *C. elegans*, *P. pacificus* is not a strict bacteriovorous nematode, because it can also feed on fungi and predate on other nematodes. It forms teeth-like denticles that are absent in *C. elegans* and other rhabditid nematodes [22]. Interestingly, the formation of teeth in *P. pacificus* is an example of phenotypic plasticity with the possibility to develop two alternative mouth morphologies (morphs), called the eurystomatous (Eu) and stenostomatous (St) forms [23]. This developmental decision is irreversible with several characteristics defining the two different morphs. In the Eu morph, this includes a wider buccal cavity and the presence of two hooked teeth (figure 1*a*). These adaptations facilitate an expanded dietary range as in addition to feeding on bacteria these teeth can be employed to penetrate the cuticle of other nematodes including *C. elegans*. On the contrary, St morphs possess only a single flint-shaped tooth, and as such they are not capable of preying on other nematodes and feed on only microbes (figure 1*b*) [24–26].

Mouth-form plasticity is influenced by environmental (conditional) and stochastic factors. First, environmental cues such as starvation, pheromones and crowding [27,28], as well as nutritional status related to culture and growth conditions [29,30], feed into the genetic network controlling mouth-form plasticity. It is therefore feasible that as cilia play an essential role in the uptake of many environmental cues, they may also provide an entry point for information necessary for the developmental decision controlling the *P. pacificus* mouth-form dimorphism. Second, mouth-form plasticity also shows stochastic regulation. For example, in the wild-type strain *P. pacificus* PS312 more than 70% of animals in standard laboratory culture conditions with *Escherichia coli* OP50 as food are Eu, whereas the rest of the animals develop into the St morph [31,32]. This stochastic aspect in mouth-form regulation has been considered to represent an example of a bet-hedging strategy, previously shown in many microbial species that live in highly fluctuating and partly unpredictable environments [33,34].

An additional environmental input necessary for the predatory nematode *P. pacificus* is the detection of potential prey. This behaviour is dependent on an interaction between the nose of the predator and the cuticle of the prey, which induces a behavioural switch instigating a predatory feeding mode [25,35]. In addition to the detection of potential prey, it has recently been shown that *P. pacificus* predators are also capable of distinguishing self-progeny from non-self [36]. While so far only a signalling ligand involved in this process has been identified, the ciliated head sensory neurons are primary candidates for the associated receptors.

Here, we phenotype a wide variety of cilia-related mutants previously isolated in *P. pacificus*, to assess the role of cilia in the regulation of the mouth-form dimorphism and predatory behaviour. This set of mutants includes representatives of all six IFT subcomplexes [19] (see electronic supplementary

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material, figure S1) plus the regulatory factor X (RFX) transcription factor *daf-19*, the master regulator of ciliogenesis in nematodes [21,37–39].

2. Methods

(a) Strains

Wild-type *P. pacificus* strains and mutant lines used in this study are listed in electronic supplementary material, table S1. Strains were maintained at 20°C using standard methods [40].

(b) CRISPR/Cas9 mutagenesis

Procedures for CRISPR-Cas9 mutagenesis followed the existing protocol for *P. pacificus* [41]. sgRNAs (electronic supplementary material, table S2) were synthesized by Integrated DNA Technologies Inc. (Coralville, IA, USA) and Cas9 protein was produced by New England BioLabs Inc. (Ipswich, MA, USA). Injection Master Mix was prepared following the manufacturer instructions from Alt-RTM CRISPR-Cas9 System User guide from Integrated DNA Technologies Inc. Injections were performed on a Zeiss Axiovert microscope (Zeiss, Germany) coupled to an Eppendorf TransferMan micromanipulator and Eppendorf FemtoJet injector (Eppendorf AG, Hamburg, Germany). Injected P0 animals were kept individually on NGM plates for 16 h. Around 100 F1 progeny were isolated onto NGM plates before they became adults. Once they had laid eggs, F1 individuals were lysed and assayed for the presence of a molecular lesion around the sgRNA target site by PCR and subsequent Sanger sequencing. Primer sequences and PCR conditions for detecting molecular lesions are listed in electronic supplementary material, table S3.

(c) Genetics

daf-19 and *osm-1* single mutants were crossed with the *nag-1 nag-2* double mutant [32] in order to produce triple mutants to study the epistatic relationships between those genes. Molecular lesions in both triple mutants were confirmed by PCR and subsequent Sanger sequencing. Primer sequences and PCR conditions for detecting molecular lesions are listed in electronic supplementary material, table S3.

(d) Mouth-form phenotyping

Mouth-form phenotyping was done in three culture conditions: First, standard culture conditions for *P. pacificus* on NGM agar plates seeded with *E. coli* OP50, which induce the Eu morph in wild-type strains [23]. Second, NGM agar cultures with the bacterial isolate *Pseudomonas* sp. LRB26 as food source, which increases the ratio of Eu animals in wild-type strains even further [30,42]. Third, standard liquid cultures in S-medium, which strongly represses the Eu morph in wild-type strains [29]. For the first two conditions, five young adult hermaphrodite individuals were transferred onto 6 cm NGM agar plates seeded with 300 µl of the corresponding bacteria strain from the same well-fed source plate. These plates were incubated at 20°C for 5 days. For liquid culture conditions, media were prepared following the protocol from Werner *et al.* [29] and 10 young adults were employed to initialize the culture. Liquid cultures were incubated at 22°C and shaken at 180 r.p.m. (INFORS HT Multitron standard) for 6 days. After the corresponding incubation time, young adult hermaphrodite individuals were transferred from the corresponding culture plate or flask to 4% agar pads (containing 10 mM sodium azide) with 5–8 µl M9 buffer and mouth-form phenotypes were observed and scored under a differential interference contrast (DIC) microscope (Zeiss Axioskop) using 10× and 100× magnifications. Several discrete characters were used to discriminate between Eu and St individuals, such as the presence versus absence of a subventral tooth and the width of the buccal cavity. Morph frequencies were calculated from three biological replicates per line. Thirty young adult hermaphrodite individuals were examined per replicate.

(e) Predatory behaviour and self-recognition phenotyping

Predatory feeding behavioural assays were carried out using previously described corpse and biting assays [25,36], which are summarized as follows.

(i) Corpse assays

For assays using either *C. elegans* or *P. pacificus* as prey, the prey animals were maintained on bacteria until freshly starved, resulting in an abundance of young larvae. These plates were washed with M9 buffer, passed twice through a 20 µm filter, centrifuged and deposited onto the assay plate by pipetting 1 µl of worm pellet onto a 6 cm NGM unseeded plate. This resulted in roughly 3000 prey larvae on each assay plate. For *C. elegans* assays, five *P. pacificus* predators of the control strain (PS312) or a cilia mutant strain were screened for the appropriate predatory Eu mouth morph and these were subsequently added to assay plates. Predators were permitted to feed on the prey for 2 h before removal and the plate screened for the presence of corpses. For self assays using *P. pacificus* prey, 20 *P. pacificus* predators were added to each assay plate and left with the prey for 24 h, because intra-specific killing is known to be less frequent than inter-species killing [36]. Each assay was replicated five times.

(ii) Biting assays

In order to observe the interaction between predator and prey directly, bite assays were carried out as follows. *C. elegans* or *P. pacificus* prey was added to assay plates as previously described for corpse assays. The appropriate *P. pacificus* nematodes were screened for the required predatory Eu mouth morph and a single predator placed on to the assay plate and allowed to recover for 20 min. After recovery, the predatory animal was observed for 10 min and the number of bites quantified. Bites were characterized by a switch in predator pumping mode coinciding with a restriction in movement of the prey. Each assay was conducted with 10 different animals.

(f) Statistical analyses

Statistical analyses were performed in the computing environment R v.3.4.2 (R Core Team, 2017). Replicates were used to calculate means and standard errors (s.e.m.). Morph frequencies were compared by fitting beta regression using the R package betareg [43]. Given the fact that morph ratios in some strains included the extremes 0 and 1, the transformation $(y(n-1)+0.5)/n$ was applied to the data, where y is the response variable and n is the sample size [44]. Replicates within lines were included in the model, and significance was assessed using a z-test. *Post hoc* pairwise comparisons between lines and biological replicates were performed by the least-squares means method from the R package lsmeans and significance thresholds were set using the false discovery rate correction method [45]. All p-values < 0.05 are summarized with asterisks in corresponding figures.

3. Results

(a) *P. pacificus* IFT and *daf-19* mutants show a diverse range of mouth-form ratios

The mouth-form dimorphism of *P. pacificus* is influenced by environmental cues, which are probably sensed through the

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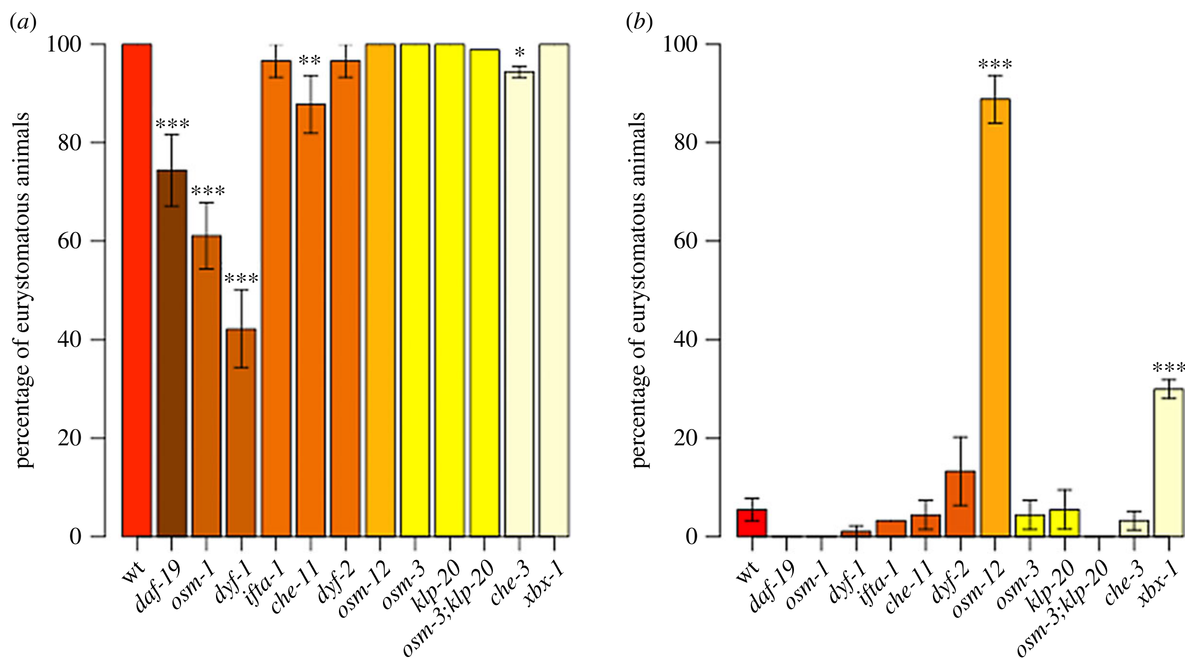

Figure 2. (*a*) Mouth-form ratios presented as percentage eurystomatous for hermaphrodites of wild-type, *daf-19* and IFT mutants in NGM agar cultures with the Eu mouth-form inducing *Pseudomonas* sp. LRB26 as food source. (*b*) Mouth-form ratios presented as percentage eurystomatous for hermaphrodites of wild-type, *daf-19* and IFT mutants in the St mouth-form inducing standard liquid culture. For the IFT mutants, in both subfigures, colours indicate the IFT subcomplex to which the IFT genes belong (see figure 1*c*; electronic supplementary material, figure S1). In all experiments, three biological replicates were performed for each line, and 30 young adult individuals were examined per replicate. Error bars represent the standard error of the mean. $^{*}p < 0.05$; $^{***}p < 0.001$; all the comparisons are to wild-type and only statistically significant comparisons are shown (electronic supplementary material, tables S4B and S4C). (Online version in colour.)

cilia of sensory neurons. To analyse the role of cilia in the regulation of mouth-form plasticity we have determined the mouth-form ratio of the IFT and *daf-19* mutants previously isolated in *P. pacificus* [19,21] under standard NGM agar culture conditions. Surprisingly, we found a wide range of mouth-form ratios among the different mutant alleles, from mutants with a high proportion of St animals to mutants with a high proportion of Eu animals (figure 1*c*; electronic supplementary material, table S4A). The two mutant lines showing the strongest St phenotype were *daf-19* and *osm-1* mutants, followed by the *osm-3; klp-20* double kinesin mutant, the *che-3* and the *dyf-1* mutants. *ifta-1* mutants showed an intermediate mouth-form rate, while *che-11* mutants showed a slight reduction in the proportion of Eu animals. Interestingly, *dyf-2*, *osm-12*, *osm-3* and *klp-20* mutants were highly Eu similar to wild-type animals. Thus, we observed an unexpected heterogeneity of mouth-form phenotypes in cilia mutants in *P. pacificus*. Additionally, consistent with the idea that the RFX/DAF-19 transcription factor is the master regulator of nematode ciliogenesis, the *daf-19* mutant showed one of the strongest phenotypes.

(b) Culture conditions influence the mouth-form ratio of *P. pacificus* independently of cilia

As it has previously been shown that several growth conditions affect the ratio of mouth morphs in *P. pacificus* [29,30], we tested whether the information from these conditions is integrated through the sensory cilia of the nematodes. To answer this question, we determined the mouth-form ratios of nematodes grown on NGM agar plates using the bacterium *Pseudomonas* sp. LRB26 as a food source, a culture condition that increases the ratio of the Eu morph in wild-type animals [30,42]. Under these conditions, the ratio of the Eu morph increased in all tested mutants (figure 2*a*). However, not all mutations affected the phenotype to the same degree (figure 2*a*; electronic supplementary material, table S4B). For example, *ifta-1*, *che-11*, *osm-3; klp-20* and *che-3* mutants reached a percentage of Eu animals close to 100%. By contrast, the *daf-19*, *osm-1* and *dyf-1* mutants showed between 40% and 75% of Eu animals.

Similarly, we have studied the mouth-form ratio of IFT and *daf-19* mutants in liquid culture conditions that stimulate the St morph in wild-type animals [29]. In this case, all the mutants except *osm-12* showed a strong reduction in the number of Eu animals (figure 2*b*; electronic supplementary material, table S4C). These experiments suggest that the mechanism that triggers the development of the St morph in standard liquid culture conditions does not involve the cilia. Surprisingly, *osm-12* was the only IFT mutant that remained highly Eu in these culture conditions. Therefore, we generated an additional allele to confirm this result. Indeed, this new *osm-12* mutant also showed a similar high proportion of Eu animals in standard liquid culture conditions (electronic supplementary material, figure S2 and table S5). Thus, loss-of-function mutations in the different IFT components may alter the overall function of cilia in various ways, resulting in opposite mouth-form phenotypes.

(c) Cilia act upstream of mouth-form plasticity switch genes

Previous studies identified several members of the genetic pathway regulating the mouth-form plasticity of *P. pacificus*, with several significant components located in a multi-gene locus containing two sulfatases and two a-N-acetylglucosaminidases (*nag*) [32]. While one sulfatase-encoding gene, *eud-1*, acts as a developmental switch with animals carrying mutations in this

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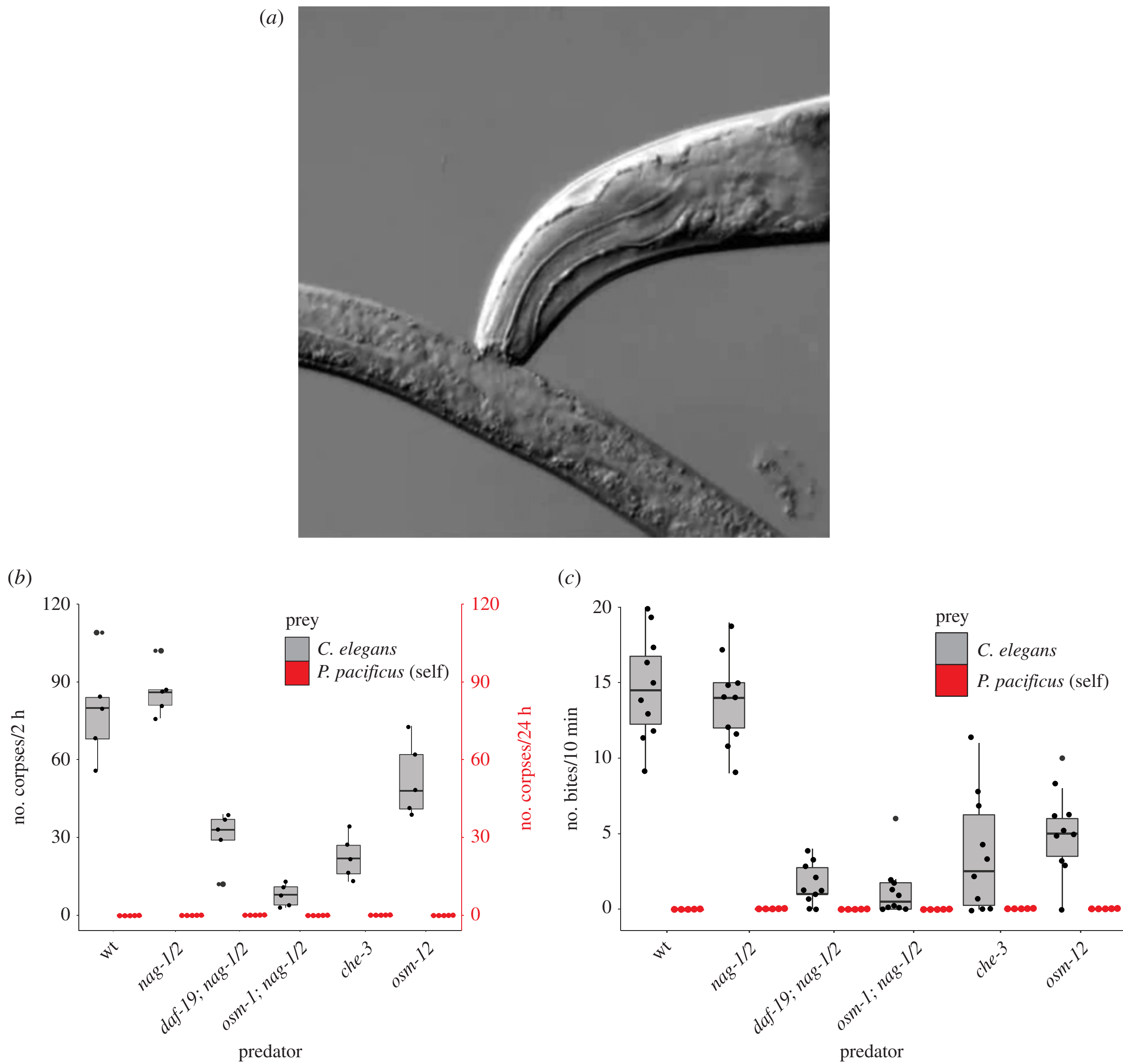

Figure 3. (*a*) Adult *P. pacificus* feeding on a larva of *C. elegans*. (*b*) Corpse assays with *P. pacificus* fed upon either *C. elegans* larvae (black) with five predators over 2 h or *P. pacificus* larvae (red) with 20 predators over 24 h. Data shown as median, first and third quartile and data range. In all experiments, five replicates were quantified for each mutant. (*c*) Biting assay feeding *P. pacificus* predators on either *C. elegans* larvae (black) or *P. pacificus* larvae (red). The number of bites was quantified during 10 min for 10 different animals of each mutant. (Online version in colour.)

gene showing all-St mouth-forms [31], the *nag* genes have an opposing effect as animals carrying mutations in both *nag* genes (*nag-1 nag-2*) form all-Eu morphs [32]. To unravel the relationship between the cilia and the genetic regulatory pathway of the mouth-form dimorphism known to date, we performed epistasis tests by creating a *daf-19; nag-1 nag-2* and an *osm-1; nag-1 nag-2* triple mutant, which were phenotyped in standard culture conditions. In both triple mutants, the proportion of Eu animals was 100%, phenocopying the *nag-1 nag-2* double mutant (electronic supplementary material, figure S3A) [32]. In addition, both triple mutants remained 100% Eu when grown in standard liquid cultures and in NGM agar cultures with *Pseudomonas* sp. LRB26 as food source (electronic supplementary material, figures S3B and S3C, and table S6). Taken together, these results suggest that the developmental switch genes regulating mouth-form act downstream of cilia. While we speculate that cilia act in sensory neurons involved in perceiving environmental input, we can currently not rule out that they might act in other cell types.

(d) Cilia mutants are partially defective for prey detection but self-recognition is maintained

The predatory behaviour of *P. pacificus* can be triggered upon nose contact with another non-self nematode. Specifically, a small peptide-mediated self-recognition code prevents killing of kin (figure 3*a*) [25,36]. As nose contact appears to be required for detection and analysis of potential prey, we hypothesized that detection and prey selection is dependent on cilia. Therefore, we tested the ability of several cilia-related mutants to predate on *C. elegans* larvae and additionally, their ability to predate on *P. pacificus* wild-type larvae. Specifically, we analysed predatory feeding behaviour in *daf-19; nag-1 nag-2* and *osm-1; nag-1 nag-2* triple mutants and the *che-3* and *osm-12* mutants by means of standard corpse assay and bites assay [25] (see Methods for assay conditions). Only these single and triple mutant combinations resulted in Eu animals necessary to perform predation assays as St animals are strictly bacteriovorous and lack predatory feeding behaviour. In all

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mutants analysed, we found a significant reduction in the predatory behaviour when feeding on *C. elegans* in comparison with wild-type animals (figure 3*b*,*c*). However, similar to our mouth-form frequency observations, we detected different degrees of predation defects among the mutants, and in no mutant condition was killing completely abolished. These findings suggest that some prey detection mechanism is independent of cilia and that mutations in the different IFT proteins and the RFX transcription factor may cause specific predatory deficiencies according to the role each of these elements play in the overall functioning of the cilia. Despite this, self-recognition was maintained in all the cilia deficient mutants with no killing of self-progeny detected (figure 3*b*,*c*), indicating that the mechanisms triggering self-recognition may not require the presence of fully functional cilia.

4. Discussion

Nematodes respond to a multitude of environmental cues in order to adapt to the fluctuating ecological conditions that they experience in nature. These cues are integrated into the neural circuitry through sensory neurons, which in many cases also develop cilia as the main cell structure for environmental sensing and signal transduction. As a consequence of this, two kinds of responses are produced: adaptive behaviours (such as food searching, predatory behaviour or escaping from noxious conditions) and developmental decisions that influence morphological plastic traits (such as the mouth-from dimorphism displayed by *P. pacificus* nematodes). Here, we have analysed the role of cilia in the regulation of mouth-form plasticity, predatory behaviour and self-recognition in *P. pacificus*.

Through our investigation, we were able to demonstrate that cilia play an important role in the regulation of mouth-form plasticity, with this developmental decision likely to depend on environmental cues sensed through these structures. The mouth-form phenotypes in the cilia defective mutants show a range of severity with significantly stronger effects observed in mutants belonging to the IFT-B subcomplex and in the RFX transcription factor *daf-19*. Previous studies have showed that *daf-19* is essential for ciliogenesis in *C. elegans* [39] and this is conserved in *P. pacificus* nematodes, with EM sections showing *Ppa-daf-19* mutants also lack cilia projections in amphid neurons [21]. Differences in the mouth-form phenotype between mutants of the IFT-B and IFT-A subcomplexes may be related to the different roles both subcomplexes play in the overall functioning of the cilia. Previous studies have shown that the IFT-B subcomplex is required for anterograde transport from the base to the tip of the cilium, while the IFT-A subcomplex functions in the retrograde movement from the tip back to the base of the cilium (reviewed in [46]). Therefore, the stronger mouth-form phenotypes of IFT-B mutants (*osm-1* and *dyf-1*) in comparison with IFT-A mutants (*ifta-1*, *che-11* and *dyf-2*) may be indicative of a greater dependence on IFT-B and the anterograde transport system for sensing of the external cues influencing the mouth-form decision. An alternative possibility is that for some individual IFT proteins, specific roles outside of their associated complexes may exist and may have distinct effects on the mouth-form phenotype. For instance, *osm-1* appears to play an essential role regulating the transition from anterograde to retrograde IFT at the flagellar tip in *Chlamydomonas* [47,48] and *dyf-1* is required for tubulin post-translational polyglutamylation in sensory neuron cilia in *C. elegans*, while its homologue *flr* performs the same function in zebrafish [49]. On the other hand, it has been reported that the inactivation of the *che-11* zebrafish homologue IFT140 had little observable effect on cilia structure, indicating some degree of functional redundancy among different IFT polypeptides [50].

Other elements involved in anterograde transport are the heterotrimeric and homodimeric kinesin-2 motors [51]. While the single kinesin mutants *osm-3* and *klp-20* did not show a mouth-form phenotype, the double kinesin mutant *osm-3; klp-20* showed a strong reduction in the percentage of Eu morphs. This phenocopying of the other anterograde defective mutants supports a functional redundancy between the heterotrimeric kinesin-II and the homodimeric OSM-3-kinesin motors, which was previously proposed for *P. pacificus* [19] and described for *C. elegans* [51]. Finally, the dynein motor subunit CHE-3 mutant showed a strong mouth-form phenotype, while the mutant of the dynein light intermediate chain XBX-1 showed no mouth-form phenotype in NGM agar culture conditions. This difference in phenotype among dynein subunits could be related to the fact that the *xbx-1* mutant carries an in-frame mutation, or alternatively that these two dynein subunits perform specific functions giving rise to different phenotypes when mutated. This second hypothesis is supported by the fact that the in-frame mutation of the *xbx-1* mutant does induce the bordering and clumping behaviours in response to hyperoxic conditions [19]. In addition, the *xbx-1* mutant showed a significantly higher proportion of Eu animals in comparison to wild-type animals in standard liquid culture conditions, suggesting *xbx-1* may play a specific role promoting the St morph under certain environmental conditions.

While our data clearly indicate an essential role for cilia in environmental sensing influencing the mouth-form, our experiments in different culture conditions imply that other additional environmental cues not sensed through cilia also regulate the mouth-form decision. This includes those produced by the different bacterial food sources or the stressful conditions associated with the standard liquid culture method. Surprisingly, the two mutant alleles of the BBSome component *osm-12* induced a high frequency of Eu morph in all culture conditions. These results indicate that disruption of the function of the BBSome promotes the Eu morph constitutively, although the reason for this is not clear. In *C. elegans*, BBS proteins play a critical role in keeping the two kinesin-2 motors together [52–54], and therefore the dissociation of these two kinesin-2 motors may alter the signalling pathway in the cilia and induce the Eu phenotype. On the other hand, the BBSome is also involved in trafficking sensory receptors into and out of the cilium in *Chlamydomonas*, *C. elegans* and mouse [55]. Thus, an impaired trafficking of sensory receptors may represent an alternative explanation for the constitutive Eu phenotype of *osm-12* mutants.

Additionally, we investigated the dependence on cilia for environmental cues specific to predatory behaviour. Defects in cilia components resulted in a reduced killing ability of *P. pacificus* predators with bite assays, confirming this was caused through a reduction in the efficiency of prey detection. While a clear reduction in killing was observed, some killing did still occur, indicating that prey detection was not fully abolished in these mutants. This may indicate that some cilia functionality remained in these mutants or that some prey recognition is independent of correct cilia formation. In accordance with these potential hypotheses, our experiments on

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self-progeny revealed no killing of identical kin, indicating self-recognition was also maintained in cilia defective mutants. Receptors involved in the efficient recognition of potential prey are therefore highly dependent on the correct development and function of cilia although not exclusively.

Altogether, our results indicate that cilia play an important role regulating the mouth-form plasticity and predation-related behaviours in *P. pacificus*. However, the various IFT components influence these traits to different degrees, which may be related to the diverse functions each of these components performs in the overall function of the cilia. While it is likely that cilia associated proteins act in neurons, they might have additional roles in other cell types. Therefore, it remains currently unknown to what extend the observed phenotypes result from neuronal cilia defects. Our overall findings are in accordance with previous studies in other organisms such as *C. elegans*, *Chlamydomonas*, *Tetrahymena* and mouse, which described specific functions for the six IFT sub-complexes [46,51–54] and some of its protein components [47–49]. Thus, sub-functionality is a conserved feature of cilia in all studied organisms. Future work will determine the environmental cue or cues sensed through the cilia that influence the mouth-form phenotype and additionally the nematode cues influencing predation behaviour. Additionally, identification of the molecular receptors and specific sensory circuits responsible for the integration of these external signals remains to be elucidated.

Data accessibility. The datasets supporting this article have been uploaded as part of the electronic supplementary material.

Authors' contributions. E.M., J.W.L., M.L. and R.J.S. designed research; E.M., J.W.L. and M.L. performed research; E.M. and J.W.L. analysed data; and E.M., J.W.L. and R.J.S. wrote the paper.

Competing interests. The authors declare no competing interests.

Funding. The work was funded by the Max Planck Society.

Acknowledgements. We thank Bogdan Sieriebriennikov for discussion and providing the *nag-1 nag-2 double* mutant and the corresponding sequencing primers. We also thank Nermin Akduman for providing *Pseudomonas* sp. LRB26.

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Co-option and developmental system drift in sensory mechanisms of developmental plasticity in nematodes

Abstract

Polyphenism, a discrete case of developmental plasticity, is a common phenomenon in nature. However, very little is known about the molecular pathways that integrate environmental signals into binary phenotypes. Receptor redundancy and a lack of molecular tools in non-model organisms are some of the issues which have hitherto hindered the research of receptive pathways. In this study, we describe the reaction norm of the mouth-form dimorphism in the nematode *Pristionchus pacificus* across a temperature gradient. Through an unbiased mutagenesis approach, we identify *daf-25*/Ankmy2 and its target *daf-11*/guanylyl cyclase as crucial genetic regulators of the induction of the bacteriovorus morph by high temperature. Through a candidate approach we identify the *tax-2*/cGMP-gated ion channel as a possible downstream target of cGMP signalling. The finding that the same pathway is also used for integration of temperature into another, dauer polyphenism in the model nematode *C. elegans* motivated us to investigate the role of *daf-11* and temperature in dauer induction in *P. pacificus*. We found that the dauer in *P. pacificus* is also temperature-sensitive, however, *daf-11* and *tax-2* mutants have contrasting dauer formation defects compared to the mutants in *C. elegans*. We further show that the expression pattern of *daf-11* is probably not conserved among the nematodes. Our study furthers the understanding of the mechanism and evolution of receptive genetic pathways which integrate the environment into developmental plasticity.

Introduction

Polyphenisms are cases of developmental plasticity involving discrete phenotypes; abundant in nature, they are particularly valuable for the investigation of genetic mechanisms of phenotypic plasticity due to the clear relationship between phenotype and environment reflected in the discrete readout (West-Eberhardt, 2003). Studies on the molecular mechanisms of the regulation of polyphenism have revealed roughly three main hierarchical levels of signal integration into the development: the reception of the signal (receptors and far upstream

signalling), the fixation of the developmental trajectory (switch genes, such as hormones and transcription regulators) and the execution of morphology/behaviour (structural genes and other downstream morph-specific genes) (Bopp et al., 2014; Trible and Kronauer, 2017).

Previous research mainly focused on the switch regulatory networks and downstream signaling pathways, whereas upstream signaling pathways which integrate environmental signals into the switch networks are the least investigated. In most organisms, inductive cues, receptor molecules and integrating tissues remain spurious (Simpson, 2011; Projecto-Garcia et al., 2017; Lafuente and Beldade, 2019; Yang and Pospisilik, 2019). So far, the most comprehensive understanding of receptive mechanisms of polyphenisms are found in locusts and *Caenorhabditis elegans*, where sensory tissues and their corresponding neural pathways, as well as specific cues, are well understood (Cullen et al., 2017; Androwski et al., 2017). These early results revealed that it is not possible to simply map previously identified receptors involved in behavioural and physiological phenotypically plastic responses to developmental plasticity. For example, in *C. elegans,* the main thermotactic neuron AFD is not crucial for dauer formation on high temperatures, a highly stress-resistant alternative larval stage (Cassada and Russell, 1975; Ailion and Thomas, 2000).

The genetic research of developmental plasticity is mainly hindered by the lack of molecular and genetic technologies in more complex organisms (Lafuente and Beldade, 2019). Therefore, simpler organisms like nematodes are useful models to address these questions. The nematode *Pristionchus pacificus* has become an established model system for evolutionary and developmental research (Sommer, 2015). *P. pacificus* shares the dauer polyphenism with *C. elegans* and has an additional polyphenism in mouth structures (Ogawa and Brown, 2015; Figure 1a). The comparison of *C. elegans* and *P. pacificus* dauer larvae has revealed the conserved role of the switch transcriptional regulator *daf-12* in both dauer polyphenisms (Ogawa et al., 2009). However, divergence in molecular regulatory pathways was observed both upstream and downstream from the switch transcription factors, presumably reflecting specific lifestyles and functional specialisations of the two dauer larvae (Hong and Sommer, 2006; Moreno, Lenuzzi et al., 2018; Cinkornpumin et al., 2014; Mayer et al., 2011; Bose et al., 2014; Mayer et al., 2015; Markov et al., 2016; Sinha et al., 2012). While *P. pacificus* dauers are commonly found on scarab beetles, *C. elegans* dauers have not been found in such host-specific relationships (Herrman et al., 2006; Hong et al., 2008; Félix and Duveau, 2012). Besides its dauer polyphenism, *P. pacificus*

also exhibits a unique mouth-form polyphenism, which enables it to exploit alternative food sources (Figure 1a). In its eurystomatous form (henceforth often simply "eu"), the worm has two movable teeth, which enable it to feed on other nematodes, while in its other, stenostomatous form (henceforth often simply "st"), it can feed only on bacteria (Bento et al., 2010; Wilecki et al., 2015). The stenostomatous form has a slight advantage in developmental speed when only bacterial food is available (Serobyan at al., 2013).

P. pacificus' mouth-form dimorphism is regulated conditionally by the environment, but there is also a stochastic regulation reflected in a constant eu : st ratio in standard conditions, which for the wild-type strain PS312 is 90 : 10 % in standard conditions (NGM agar + 200-300ul OP50) (Sommer et al., 2017). The eu form is induced in crowding conditions *via* pheromones, such as *dasc#1* and *pasc#1*, as well as some types of bacterial food from the genus *Novosphingobium,* while the switch towards preferentially st form can be pushed by nematode isolation, exposure to high salt concentrations and liquid culture condition (Bento et al., 2010; Serobyan et al., 2013; Ragsdale et al., 2013; Bose et al., 2014; Werner et al., 2017; Namdeo et al., 2018; Akduman et al., 2020). The presence of both mouth forms in standard conditions enables easy screening for mutants defective in switch genes inducing one or the other form. Indeed, forward genetic screens in *P. pacificus* revealed a complex switch network, including the master switches *eud-1* and *nhr-40*, which are necessary for the eu morph induction (Ragsdale et al., 2013; Kieninger et al., 2016; Sieriebriennikov et al., 2020). Further research revealed several other regulators, such as epigenetic factors *lsy-12* and *mbd-2*, the long non-coding RNA *as-eud-1,* which positively regulates the master switch *eud-1*, as well as the role of genes neighbouring *eud-1* in mouth-form dimorphism revealing a multigene locus consisting of two sulfatases and two NAGLU genes (Serobyan et al., 2016; Sieriebriennikov et al., 2018). Upstream signaling molecules and sensory tissues of the *P. pacificus* mouth-form dimorphism have been explored through candidate gene approaches by testing the mouth-form effect of previously generated mutants of the structural components of the sensory cilia and *daf-19*/RFX, a master regulator of development of sensory cilia, in the laboratory. The cumulative effect of the loss of the sensory cilia resulted in the increase in st. However, individual structural components of the displayed contrasting effects on the mouth-form regulation (Moreno, Lenuzzi et al., 2018; Moreno et al., 2019).

Temperature-dependent developmental plasticities have been well described in several organisms (Lees, 1959; Shapiro, 1982; Smith, 1993; Bull, 1980; Golden and Riddle, 1984; Tanaka, 2004). The research on switch molecules in several polyphenisms provides a basic framework for our understanding of the molecular mechanisms of alternative morph induction by different temperatures (Carroll et al., 1994; Reed and Serfas, 2004; Brakefield et al., 2009; Merchant-Larios and Diaz-Hernandez, 2013; Ge et al., 2017; Ge et al., 2018). Recently, the TRPV4 receptor has been identified as a regulator of male development in *Alligator mississippiensis'* temperature-dependent sex determination (Yatsu et al., 2015). Research of temperature-sensitive dauer mutants in *C. elegans* presumably provided the best molecular understanding of temperature integration into developmental plasticity. In short, high temperature is sensed by sensory neurons through cGMP signaling, communicating the signal to downstream insulin-like and TGF-beta-like signaling, in turn leading to the activation of the transcription factor *daf-16* to induce stress-resistant dauers (Ailion and Thomas, 2000; Ailion and Thomas, 2003).

In this work we describe the response of *P. pacificus'* mouth-form polyphenism to high temperature and identify the receptive signaling pathway through an unbiased mutagenic approach.

Materials and methods

Strains and culturing

The reference strain *P. pacificus* PS312 and its derivative RS2333 and wild isolates of *P. pacificus* RS5134, RS5160, RS5417, RSA622, RSA635, RSA644, RSA645, RSC016, RSC018 and RSC028 were used for the mouth-form phenotyping on 27°C. All strains were maintained in the lab and inbred for at least 8 generations. The strain RS2333 was obtained from a lab culture, while other strains were obtained from frozen stocks in liquid nitrogen at the beginning of the experiments. Forward genetic screen was performed on strain RSA635 to isolate mutants *tu714, tu715, tu716, tu717, tu718, tu719, tu720, tu721, tu722, tu723, tu724.* CRISPR/Cas9-mediated genome engineering was used to generate the following mutants:*daf-25(tu1516), daf-25(tu1517), daf-11(tu1438), daf-11(tu1439), daf-11(tu1440), tax-2(tu1296), tax-2(tu1291), tax-2(tu1292),*

daf-16(tu1514), daf-16(tu1515). The reference strain, wild-isolates and mutant lines were maintained at 20°C using standard methods (Pires daSilva, 2006).

Mouth-form phenotyping

The mouth-form phenotype was scored as described previously by Serobyan et al. (2013). Briefly, eu and st individuals were distinguished by the presence *vs.* absence of a subventral tooth and a claw-like vs flint-like dorsal tooth. Only clear phenotypes were scored. Phenotypes were mostly clear across temperatures, with slightly more intermediates on extreme temperatures as previously described (Sieribriennikov et al., 2017). Phenotyping was performed using Zeiss Discovery V.12 and V.20 stereo microscopes and interference contrast (DIC) microscopy on a Zeiss Axioskop.

Mutant screen and identification of temperature insensitive mutants

We screened for temperature insensitive mutant strains using Ethyl methanesulfonate (EMS) mutagenesis in *P. pacificus* strain RSA635. We generated approx. 1200 gametes and screened 2400 homozygous F2 lines. F2s were transferred from the 20 to 27°C in the Memmert incubators on NGM plates with 200-300ul OP50 and the mouth-form of F3 progeny was scored after 5-7 days.

Analysis of mutant whole-genome data

Raw Illumina reads were aligned against the *P. pacificus* PS312 reference genome (version El Paco) with the help of the aln and sampe programs of BWA (version 0.7.12) software suite (Li and Durbin 2010). To distinguish between mutations and variants of the different genetic background, recently generated whole genome sequencing data of the parental strain (RSA635) (McGaughran et al. 2016) was used as a control sample. To this end, differential variant calls were generated from the mpileup output of the samtools (version 0.1.18) package (Li et al. 2009) by searching for sites with at least 5X coverage where the major allele frequency was >90% but differed between the mutant and the control sample. Functional classification of differential variants was performed as described previously (Sieriebriennikov et al. 2020).

Bulk segregant analysis

For bulk segregant analysis (BSA), *tu716* and *tu722* were back-crossed once with the wildtype. We selected *tu716* and *tu722* for genetic mapping due to their good growth and stable phenotype on high temperatures. To confirm the success of the cross, *tu716* F1s were sequenced with

F primers 5'-GGGTTTTGAAATTGTGCTTTGGTAGC-3' and

5'-GTGCATTCGTGACGCAGTAAGC-3' and

Rprimers 5'-CAGTGGCAAAAGGAGATTGATATGG-3' and

5'-TGCAGAGGACAGTGGCAAAAGG-3'(UMM-S57-42.0-mRNA-1).

For tu722 F1s, F primers 5'-GAACTCTAATGCCTAGCTAAGAAGACATCG-3' and

5'-CCCAGAATAACCTCTTCAAATCATCG-3' and

R primers 5'- CATTATCAATTGCGCCGATGTC and

5'-GAATGGAAGTCATTGTCTGATGCC-3' were used (UMM-S245-6.21-mRNA-1).

All primers were obtained from Thermo Fisher Scientific. The mutations in these genes were identified in genome sequencing of the mutant lines. From each F1, four F2 were picked and transferred to 27°C. Mouth-form was phenotyped after 6 days. After one generation of recovery on 20°C, individual lines were transferred again to 27°C and mouth-form was scored in progeny after 6 days. In total, all lines were phenotyped until at least three consecutive generations showed the stable highly eu or highly st phenotype. Heterozygote lines with intermediate phenotypes were discarded from the analysis. Stable lines were sequenced using in-house single-worm Tn5 libraries. Whole genome sequencing data from recombinant lines was aligned as described above and differential variants sites between the initial mutant line and the wild-type strain RSA635 were genotyped with the help of the samtools mpileup and bcftools (version 0.1.17) programs. At each variant site, an association between genotype and phenotype was tested using a Fisher's exact test.

CRISPR-Cas9 mutagenesis

We used modified CRISPR-Cas9 protocol for *P. pacificus* described in (Witte et al., 2015) with hybridized target-specific CRISPR RNAs (crRNAs) and universal trans-activating CRISPR RNA (tracrRNA) obtained from Integrated DNA Technologies (Alt-R product line) for generating *daf-25, daf-11*, *tax-2* and *daf-16* mutants. The sgRNA and all the crRNAs targeted 20 bp upstream of protospacer adjacent motifs (PAMs). To hybridize crRNA and tracrRNA, 10 µL of the 100 µM stock of each molecule were combined, followed by denaturation step at 95°C, 5',

and annealing at room temperature for 5 min. 5 µL of the hybridization product was combined with 1 µL of 20 µM Cas9 protein (New England Biolabs), RT, 5'. The mixture was diluted with Tris-EDTA buffer to the total volume of 25 µL and injected in the gonad rachis of young adults with no or one egg visible in the gonad. Molecular lesions were detected in F1 progeny by Sanger sequencing.

Dauer assays

Worms were raised on agar plates (60mm, NGM, 300ul OP50), until they used all the food, but prior to starvation. OP50 food for the assay inoculated in LB medium, grown overnight at 30°C and then kept at 4°C. Prior to the assay, the concentration of OP50 was measured by NanoDrop. (n(liquid cultures) x 4/OD600)ml of bacteria was transferred into 50ml conical tubes and pelleted for 30’ at 4°C, 4000rpm. After centrifugation, the supernatant was discarded and OP50 pellets were resuspended in n x 9ml of S-medium + cholesterol (Stiernagle, 2006). n x 4µl of nystatine was added to the bacterial resuspension to prevent fungal growth. The feeding solution was transferred into a 250ml Erlenmeyer flask. We washed 3 mix-stage plates with S-buffer + cholesterol into a 1ml eppendorf tube using Pasteur plastic pipettes. In the case of several washes, worms were spinned down at 1000rpm, 2’ at RT, then let to settle down for additional 2-3’. We pipetted 1ml of nematodes into Erlenmeyer flasks with the OP50 feeding solution and left them at 20 or 27°C, 180rpm. After 9 days, we scored the number of dauers under Zeiss Axioscope. To facilitate the screen, worms were immobilized with 0.2% NaNO3.

Genetic transformation

To generate a reporter construct, we amplified the upstream sequence of the in-silico identified daf-11 start codon to the 3’UTR of the next gene (794bp) with primers
5'-CACCGTAAAGTCCTCCTGCAGGAACCATGACGTCATTGCAAG-3' (Fwd) and
5'-GATCAGCTCGCTCATCCACAAACCCGCTGAATTTA-3' (Rev).
We amplified TurboRFP and 3’UTR of the ribosomal gene rpl-23 with primers
5'-TCAGCGGGTTTGTGGATGAGCGAGCTGATCAAGGA-3' (Fwd) and
5'-CCAATGCATTGGTTCTGCAGTTTGTGCTGCAAGGCGATTAAGTTGGGTAA-3' (Rev) from pUC19-based plasmid stock. All primers were obtained from Thermo Fisher Scientific. Cloning was done using Gibson Assembly® Cloning Kit (New England Biolabs). Cloning product was amplified as a linear construct using primers

5'-CACCGTAAAGTCCTCCTGCAGGAACCATGACGTCATTGCAAG-3' (Fwd) and 5'-CCAATGCATTGGTTCTGCAGTTTGTGCTGCAAGGCGATTAAGTTGGGTAA-3' (Rev) and confirmed by Sanger sequencing. For all amplification steps we used PrimeStar GxL polymerase (Takara). Prior to injection, linear construct and genomic carrier DNA of RSA635 were incubated 2h with PstI (New England BioLabs). The construct (60 ng/µl) was injected with the co-injection marker egl-20promoter::TurboRFP (10 ng/µl) and genomic DNA (1800 ng/µl) to one or both gonad arms in early adults. Images were acquired using Leica TCS SP8 confocal system and were analyzed using ImageJ.

Statistical analysis

Due to unsynchronized sampling of mutant and wild-type lines, all replicates of mouth-form phenotype mutant lines and wild-type RSA635 were combined and compared using Fisher's exact test. p-values were corrected using the FDR method. All mutant lines and wild-type lines were regularly observed over the course of several months due to possible fluctuations in the phenotype over time to confirm the stability of the phenotype. See Supplementary materials for complete statistical analysis (Table S1-7). For clearer display, the barchart in Figure 3b contains total numbers converted into percentage with the each within-line replicate following the order of collection. p-values are added above the relevant column (‘*’ <0.05, ‘**’ <0.01, ‘***’ <0.001, ‘ns’ non-significant).

Results

Wild isolates of *P. pacificus* show strain-specific temperature sensitivity

To study the molecular mechanisms of environmental perception that control mouth-form plasticity in *P. pacificus*, we wanted to investigate the influence of temperature as an abiotic factor for several reasons. First, temperature is known to be involved in the regulation of plastic traits in a diversity of animals and plants (Pigliucci, 2001). Second, the molecular mechanisms of temperature sensing in development and physiology have been investigated in several model organisms. This includes comprehensive investigations in *C. elegans* (Aoki and Mori, 2015). Finally, *P. pacificus* exhibits higher temperature tolerance than *C. elegans* with several tested wild isolates being capable of reproducing at 30 °C (Leaver et al., 2016). Thus, studying the

mechanisms of temperature perception during mouth-form regulation in *P. pacificus* might be able to identify the conservation and potential divergence of the molecular mechanisms involved in temperature sensation.

First, we investigated mouth-form ratios after growing the wild type strain *P. pacificus* PS312 at 10 °C, 15 °C, 20 °C, 25 °C and 27°C on standard NGM agar plates (Figure 1b). Temperature affected the mouth-form ratio; however, at all temperatures we observed more than 80% Eu animals (Figure 1c). Thus, in contrast to liquid culture that basically reverts mouth-form ratios from preferentially Eu to preferentially St mouth forms, temperature has a limited effect on mouth form in *P. pacificus* PS312-derived laboratory strain RS2333 (Werner et al., 2017). This might result from original adaptations of the RS2333 genotype or from domestication processes. It should be noted that the PS312 strain has been isolated in Pasadena, California in 1988 and is in laboratory culture ever since, usually at 20 °C or 15 ° C (Sommer et al., 1996). Therefore, we next investigated 10 natural isolates of *P. pacificus* that cover the large diversity of *P. pacificus,* including several isolates from the tropical La Réunion Island in the Indian ocean (Morgan et al., 2012; Rödelsperger et al., 2014). Importantly, none of these strains has undergone a long period of domestication in the lab and most of them have been frozen in the first 20 generations after isolation. Indeed, testing the same set of temperatures in these strains revealed massive influence of temperature on mouth-form ratios (Figure 1c). Specifically, many strains show a bell-like curve with the highest amount of Eu animals observed at 20 °C, such as in RSA622, RSA635 and RSA645. In other strains, the highest Eu ratios are found at 15 °C or 25 °C culture conditions. Additionally, some strains, like RS5160, do not form more than 50% Eu animals at any temperature. We conclude from these findings that the large majority of *P. pacificus* strains exhibit strong temperature sensitivity, largely in a strain-specific manner. Thus, temperature affects mouth-form plasticity and can likely be subjected to genetic investigations.

Forward genetic screens in strain RSA635 identify temperature sensing-deficient mutants

To initiate genetic analysis of temperature perception of *P. pacificus* mouth-form plasticity, we selected the strain RSA635 with its bell-shaped temperature response curve. From all tested strains, RSA635 shows one of the strongest temperature responses. At 20°C, around 80% of the animals have an Eu mouth form, whereas less than 15% are Eu at the two extreme

temperatures 10°C and 27°C (Figure 1c). We performed EMS mutagenesis with RSA635 J4 juveniles using the standard *P. pacificus* mutagenesis protocol (Aurilio and Srinivasan, 2015).

Subsequently, we tested the progeny of 2400 F2 clonal lines for high Eu mouth-form ratios after culturing the F3 generation at 27°C. We expected two potential classes of mutants with elevated Eu mouth-form ratios. First, mutant lines that affect the mouth-form switch might result in complete or nearly-complete Eu ratios. From the previous analysis of the influence of liquid culture (Werner et al., 2017; Sieriebriennikov et al., 2018), we would hypothesize that such mutants are temperature-independent. Second, mutant lines that would affect, or even eliminate temperature perception would show increased levels of Eu animals at 27 °C, but not a complete switch to all-Eu phenotypes at all temperatures. Indeed, from our screen of 2400 F2 clonal lines, we isolated 13 mutant candidates with >70% Eu animals at 27 °C. After re-testing, 11 mutant lines with elevated Eu mouth-form ratios were recovered (Table 1, Table S1). One of them, RS3410, however, showed high variability in mouth-form ratios.

Three of these 11 mutant lines, *tu6120*, *tu6121* and *tu6122*, were more than 98% Eu at 27 °C (Table 1, Table S2). This phenotype is similar to the phenotype of mutations in the *nag-1* and *nag-2* genes in *P. pacificus* PS312, which are part of the multi-gene switch locus (Sieriebriennikov et al., 2018). To test if these mutant lines affect the mouth-form switch, we phenotyped them at 20 °C and performed whole genome sequencing (WGS) to identify potential mutations in candidate genes. Indeed, all three mutant lines were also close to 100% Eu at 20 °C and thus, the effect on mouth-form ratio is temperature-independent (Table 1, Table S1). Additionally, WGS identified mutations in *nag-1* in all three mutant lines. Specifically, *tu724* has a nonsense mutation, whereas *tu715* and *tu719* result in nonsynonymous changes in *Ppa-nag-1*. Together, these experiments indicate that mutants with altered temperature perception during plasticity regulation can be isolated in *P. pacificus* RSA635. Furthermore, they support the idea that the multi-gene locus controlling mouth-form switching is conserved among strains and acts in a temperature-independent manner.

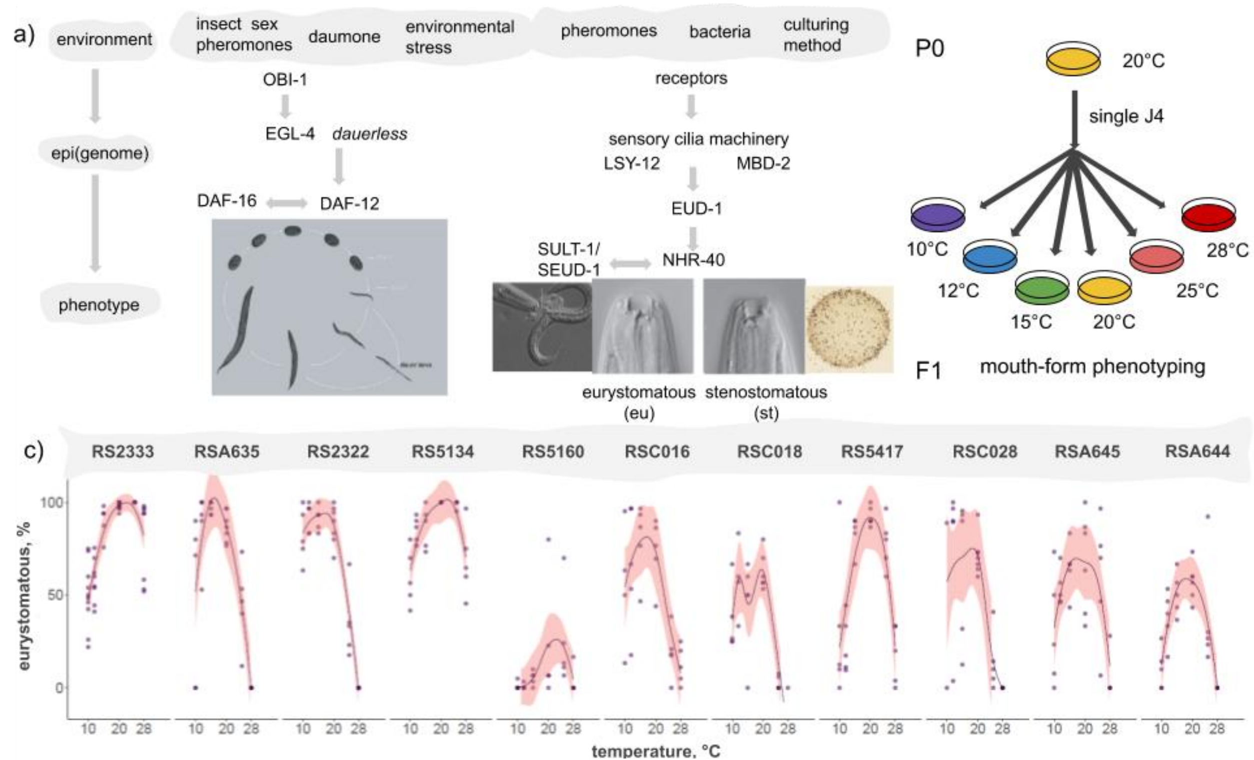

Figure 1: mouth-form polyphenism in *P. pacificus* is affected by temperature. a) dauer and mouth-form dimorphism in *P. pacificus* with schematic representations of regulatory mechanisms; b) experimental set up for a mouth-form screen on different temperatures; c) natural variation in the reaction norm of *P. pacificus* dimorphism across 10°C to 28°C.

tu716 is caused by a mutation in *daf-25*, an ortholog of mammalian Ankmy2

From the remaining nine mutant lines with elevated Eu mouth-form ratios at 27 °C, we selected two lines for further analysis. These mutants, *tu716* and *tu722*, showed the most consistent phenotype over multiple generations. For example, *tu716* is 99% Eu at 27 °C and 90% Eu at 20 °C (Table 1). We mapped *tu716* through a modified bulk segregant analysis regime (see Materials and Methods for details). In short, we sequenced 23 mutant animals after backcrossing with the RSA635 wild type. We generated Tn5 single-worm libraries for all 23 mutant animals, and a similar number of non-mutant control animals resulting from the same cross. Using unique adaptors for all animals, we performed sequencing on the HiSeq 3000 platform. When we analyzed for SNP enrichment in the mutant batch, we identified a potential mutation in the *P. pacificus* ortholog of *daf-25*. In *C. elegans*, *daf-25* mutants show a temperature-sensitive Daf-c phenotype (Jensen et al., 2010). *daf-25* is localized in cilia of sensory neurons and encodes the

ortholog of the mammalian Ankmy2, a MYND domain protein of unknown function (Jensen et al., 2010). *tu716* exhibits a missense mutation in the predicted protein pocket of *Ppa-daf-25* resulting in a V > E amino acid change (Kelley et al., 2015). To verify that the temperature sensing defect of *tu716* is indeed due to the mutation in *Ppa-daf-25*, we generated additional alleles by CRISPR knockout (Witte et al., 2015). We isolated two CRISPR-induced mutants in *Ppa-daf-25* using a guide RNA in the fourth exon, close to the site of the mutation in the original *tu716* allele. The new alleles, *tu1516* and *tu1517,* have an 11 bp insertion and an 8 bp deletion, respectively. Both alleles are highly Eu at 27 °C, similar to *Ppa-daf-25(tu716)* (Table 1). Therefore, we conclude that *Ppa-daf-25* is involved in temperature perception of mouth-form plasticity. These results are important because in *C. elegans daf-25* is known to be required for the proper localization of the guanylyl cyclase DAF-11 in cGMP signaling in cilia (Jensen et al., 2010).

daf-11/guanylyl cyclase mutant is high temperature sensitive defective

Next, we used a similar mapping strategy for the second mutant with a strong temperature-sensing defect. *tu722* is 94% Eu at 27 °C and 77% Eu at 20 °C (Table 1). Using the same bulk segregant analysis regime we found a mutation in *Ppa-daf-11* in exon 18, resulting in a premature stop codon. This observation would be consistent with the results of *Ppa-daf-25* described above. Therefore, we used a CRISPR knockout approach to confirm this observation by generating additional mutants in *Ppa-daf-11*. We used one sgRNA to induce mutations close to the N-terminus of the gene in exon 3 and a second sgRNA in exon 18, the same exon that harbors the *tu722* mutation. The sgRNA in exon 3 resulted in a single frameshift mutant F2 line with a 5 bp deletion in *Ppa-daf-11*. However, this line was sterile so that no homozygous mutant line could be generated. In contrast, we isolated four viable alleles with mutations in exon 18. *Ppa-daf-11(tu1438)* and *Ppa-daf-11(tu1439)* have 2 bp deletions and *Ppa-daf-11(tu1440)* has a 4 bp insertion. All three of these mutant lines show strongly elevated Eu mouth-form frequencies at 27 °C, similar to the original *tu722* allele (Table 1). The last allele *Ppa-daf-11(tu1443)* has a 3 bp deletion, but also results in a high Eu frequency of 87% at 27 °C (Table 1, Table S1, 2 and 3). Thus, the guanylyl cyclase *Ppa-daf-11* is involved in temperature perception during mouth-form plasticity regulation. The fact that *Ppa-daf-11* and *Ppa-daf-25* have similar phenotypes is consistent with the previously described role of *Cel*-DAF-25 in the proper localization of *Cel*-

DAF-11 to cilia in *C. elegans* (Jensen et al., 2010). Together, these findings result in two major conclusions; (i) cGMP signaling involving *Ppa-daf-11* and *Ppa-daf-25* is required for temperature sensitivity of mouth-form plasticity in *P. pacificus,* and (ii) the *daf-11/daf-25* module has been co-opted during nematode evolution for regulating the influence of temperature on mouth-form plasticity in *P. pacificus*.

***Ppa-daf-11* is expressed in multiple amphid neurons**

In *C. elegans*, *daf-11* mutants exhibit defects in several chemosensory responses, including the attraction to volatile odorants such as isoamyl alcohol (Vowels and Thomas, 1994). Also, *Cel-daf-11* mutants are Daf-c, forming dauer larvae in the absence of dauer-inducing conditions (Riddle et al., 1981; Thomas et al., 1993). Consistent with most of these phenotypes, *Cel-daf-11* is expressed in the five pairs of amphid neurons ASI, ASJ, ASK, AWB and AWC (Birnby et al., 2000). To study if the co-option of *Ppa-daf-11* for temperature perception during mouth-form plasticity regulation involved novel expression patterns, we generated a *Ppa-daf-11::rfp* reporter. We used a 794 bp promoter fragment and obtained two transgenic lines tuEx329 and tuEx330 with 30% and nearly 100% transmission rate, respectively. *Ppa-daf-11::rfp* is expressed in five pairs of amphid neurons (Figure 2a). According to the recently studied neuroanatomy of the chemosensory system of *P. pacificus* (Hong et al., 2019), these cells are AM8/ASJ, AM1/ASH, AM3/AWA, AM5/ASE and AM4/ASK (Figure 2a). These assignments suggest similarity and divergence of *daf-11* expressing amphid neurons, a finding that is similar to previous observations, such as for *Ppa-odr-7* (Hong et al., 2019). Interestingly, several genes show "switched" expression: *Ppa-daf-11* and *Ppa-odr-3* are labeling the AWA homolog rather than the AWC equivalent cell.

***Ppa-tax-2* but not *Ppa-daf-16* is required for the temperature effect on mouth form**

To identify additional components of the gene regulatory network involved in temperature perception, we used a candidate gene approach and targeted genes through CRISPR-mediated gene knockouts. First, we selected the FOXO transcription factor *daf-16* because it is known as a central regulator of many developmental and physiological processes including dauer formation in *C. elegans* (Kenyon, 2010). The analysis of *Ppa-daf-16* for a potential role in mouth-form plasticity is important because previous studies did not find any mouth-form associated

phenotype. While *Ppa-daf-16* mutants are Daf-d, like in *C. elegans*, no change in mouth-form ratios was observed in these mutants (Ogawa et al., 2011). However, these mutants were isolated in the *P. pacificus* PS312 background, which shows little temperature response as indicated above. Therefore, we generated novel *Ppa-daf-16* mutant alleles *tu1514* and *tu1515* in the RSA635 background. When we analyzed the mouth-form ratio of this mutant at 27 °C and 20 °C, we did not observe any difference from RSA635 wild-type animals (Table 1, Table S1 and 2). Thus, *Ppa-daf-16* is not involved in the regulation of mouth-form plasticity in *P. pacificus*.

Next, we wanted to know if *Ppa-daf-11* and *Ppa-daf-25* function through a canonical cGMP signaling pathway, or alternatively, through a different molecular network. Work in *C. elegans* indicated that different cGMP signaling pathways involve different guanylyl cyclases, which converge on the two cyclic nucleotide-gated channels *tax-2* and *tax-4* (Coburn et al., 1998; Birnby et al., 2000). Therefore, we generated *Ppa-tax-2* mutant alleles *tu1291, tu1292 and tu1296,* in the RSA635 background. Indeed, *Ppa-tax-2(tu1291), Ppa-tax-2(tu1292)* and *Ppa-tax-2(tu1296)* mutant animals show elevated Eu mouth-form ratios when cultured at 27 °C similar to *Ppa-daf-11* and *Ppa-daf-25* mutants (Table 1, Table S2 and 3). These findings suggest that *Ppa-daf-11* acts through a canonical cGMP signaling pathway in temperature perception in mouth-form plasticity regulation. We conclude that cGMP signaling involving *Ppa-daf-25*, *Ppa-daf-11* and *Ppa-tax-2* has been co-opted for the regulation of this evolutionary novelty. Co-option represents a central concept in evolutionary developmental biology with many independent examples in morphological diversification and novelty (Raff, 2012).

The dauer-associated phenotypes of *daf-11* and *tax-2* are not conserved in nematode evolution

In *C. elegans, daf-11* and *daf-25* were isolated in forward genetic screens for mutants displaying constitutive dauer formation (Daf-c) (Riddle et al., 1981; Jensen et al., 2010; Ailion and Thomas, 2000). Interestingly, however, we have not observed any spontaneous dauer formation in *Ppa-daf-25* and *Ppa-daf-11* mutants on the culturing plates, neither at 20°C nor at 27°C. Therefore, we tested whether *Ppa-daf-11* is involved in dauer formation, using a modified protocol for dauer induction in *P. pacificus*. First, we tested wild-type animals at both 20°C and 27°C and found that *P. pacificus* has the tendency to produce more dauers at higher temperatures, this trend is similar to what is seen in dauer assays in *C. elegans* (Figure 2b; Table S6). However,

Ppa-daf-11 mutant animals showed decrease in dauer formation in both conditions (Figure 2b; Table S4-6). Thus, *Ppa-daf-11* is Daf-d and the dauer phenotype is not conserved between *C. elegans* and *P. pacificus*. Note that *Ppa-daf-11* is not the first 1:1 orthologous gene in *P. pacificus* that when mutated does not form a Daf-c phenotype. *Ppa-daf-19*, the RTX master transcriptional regulator of ciliogenesis has no dauer phenotype (Moreno, Lenuzzi et al., 2018). Similarly to *Ppa-daf-11*, *Ppa-tax-2* mutants were Daf-d at both 20°C and 27°C, opposite from Daf-c in *C. elegans* (Ailion and Thomas, 2000; Table S4-6). Our results drive further on previous speculations on high divergence of dauer inducing pathways among nematodes.

Table 1:Mouth-form frequencies in wild type and mutant lines.

temperature , °C	genotype	eu, %	N
20	RSA635 wild-type	71	248
27	RSA635 wild-type	21	201
20	*tu714*	100	30
27	*tu714*	86	43
20	*nag-1(tu715)*	100	30
27	*nag-1(tu715)*	100	99
20	*daf-25(tu716)*	90	30
27	*daf-25(tu716)*	99	128
20	*tu717*	100	30
27	*tu717*	86	114
20	*tu718*	100	30
27	*tu718*	81	93
20	*nag-1(tu719)*	100	30
27	*nag-1(tu719)*	100	69
20	*tu720*	87	30
27	*tu720*	95	107
20	*tu721*	90	30

27	*tu721*	99	95
20	*daf-11(tu722)*	77	30
27	*daf-11(tu722)*	94	115
20	*tu723*	93	30
27	*tu723*	93	86
20	*nag-1(tu724)*	100	30
27	*nag-1(tu724)*	100	96
20	*daf-25(tu1516)*	100	90
27	*daf-25(tu1516)*	93	90
20	*daf-25(tu1517)*	98	90
27	*daf-25(tu1517)*	91	90
20	*daf-11(tu1438)*	78	86
27	*daf-11(tu1438)*	87	63
20	*daf-11(tu1439)*	87	90
27	*daf-11(tu1439)*	80	67
20	*daf-11(tu1440)*	79	90
27	*daf-11(tu1440)*	84	90
20	*tax-2(tu1291)*	95	60
27	*tax-2(tu1291)*	86	42
20	*tax-2(tu1292)*	85	51
27	*tax-2(tu1292)*	54	10
20	*tax-2(tu1296)*	93	60
27	*tax-2(tu1296)*	87	55
20	*daf-16(tu1514)*	43	85
27	*daf-16(tu1514)*	22	32
20	*daf-16(tu1515)*	51	84
27	*daf-16(tu1515)*	13	44

Discussion

In summary, we investigated the receptive pathways of signal integration into developmental plasticity by focussing on its manifestations in *P. pacificus*. We show that, on the one hand, the novel mouth-form polyphenism in *P. pacificus* evolved sensitivity to temperature through co-option of the canonical receptive cGMP pathway localized in sensory neurons. On the other hand, we show that the same pathway seems to have reversed its role in shaping the given temperature-sensitive dauer reaction norm in *P. pacificus* and *C. elegans* (Figure 2c).

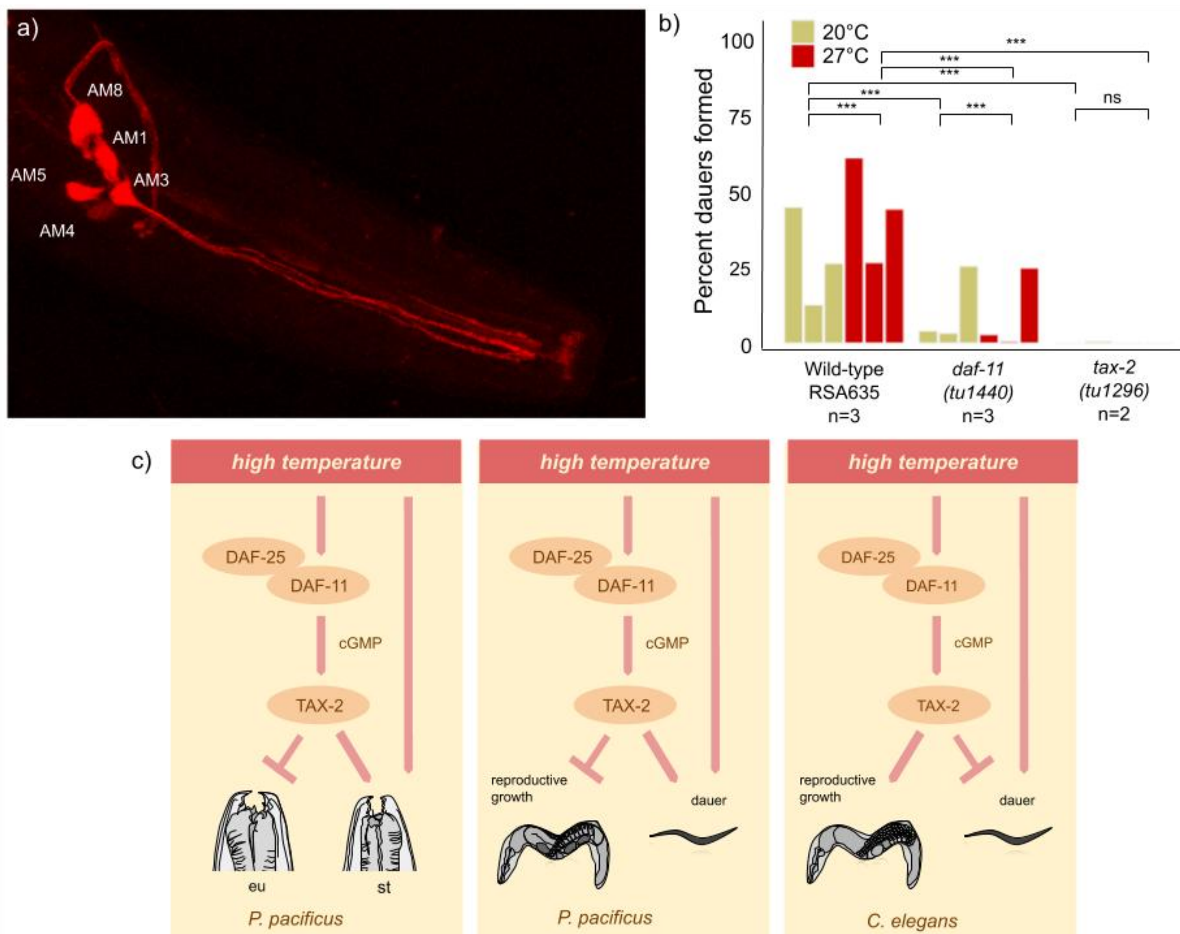

Figure 2: expression pattern of *daf-11* and its role in dauer polyphenism diverged among nematodes. a) expression pattern of *Ppa-daf-11::rfp* reporter (tuEx330), b) *daf-11* and *tax-2* mutants in *P. pacificus* are Daf-d (Fisher's exact test (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$)), c) common receptive pathway regulates integration of environmental signals into mouth-form and dauer polyphenism

Firstly, we demonstrate a novel reaction norm of mouth-form dimorphism: A U-shaped sensitivity to temperature gradient, with a gradual increase in animals developing the bacteriovorus form produced towards *P. pacificus*' lower and upper physiological limits (Figure 1c). The concave shape of the reaction norm of *P. pacificus*' mouth-form dimorphism is similar to Pattern II of temperature-dependent sex determination (TSD) in some turtles, crocodiles and lizards, where more females are produced towards the extremes, as well as to the dauer larva reaction norm in *C. elegans*, with more dauers forming in extreme temperatures (González et al., 2019; Golden and Riddle, 1984).

It remains questionable whether our experimental setup is addressing ecologically relevant phenotypes. In nature, temperatures in a single location oscillate across day-night cycles, from day to day due to weather conditions as well as with the seasons; in the context of our laboratory setting it would be very difficult to recreate the kinds of temperature oscillations a nematode in the wild would face to fully determine the role temperature plays in its development. It should be noted, however, that the significance of the differences between day and night temperatures decreases rapidly with soil depth. Possibly, *P. pacificus* can alter its mouth-form through thermotactic shifting between different layers of ground.

The complex culturing system poses the possibility that temperature indirectly influences mouth-form through the changes of bacteria or agar. While we can't exclude that the response to temperature is indirect, the strength of the response and its robustness among the wild isolates indicate that this response is important in ecological and evolutionary framework. Interestingly, high salt concentrations also lead to increased st production (Werner et al., 2017). We have observed that the reaction norm of *P. pacificus* across different salt levels also resembles a U-shaped curve (Table S7). These findings might be explained by st being a more general response to abiotic stress. This form is less complex and has shorter generation time than eu form, which might be preferred in adverse conditions (Serobyan et al., 2013). The formation of dauer larvae in nematodes is generally regarded as a response to stress, although its formation is bolstered by a wide range of environmental changes, such as heat, desiccation, crowding and starvation (Riddle, 1988; Perry and Wharton, 2011). If dauer larvae and st:eu regulation have overlapping functions in responding to stress, future comparative research on these two polyphenisms may yield further insight into the underlying evolutionary mechanisms determining the evolutionary development of novel developmentally plastic traits.

Secondly, we describe a receptive pathway which integrates environmental signals into developmental plasticity. Novel developmentally plastic traits can evolve through the evolution of novel regulatory mechanisms or through co-option of pre-existing molecules and pathways in the novel phenotype (True and Carroll, 2002; Raff, 2012). The co-option of pre-existing regulatory pathways which show environment-sensitive characteristics has been suggested to facilitate evolution of novel developmental plasticities by enabling the complex novel trait to exploit the pre-existing pathway, rather than evolving a novel one in an attempt to create a 'hopeful monster' from scratch (West-Eberhardt, 2003). Previous research revealed events of co-option of hormonal signaling, master switch molecules and sensory tissues into developmental plasticity (Brakefield et al., 1998; Moczek and Nijhout, 2002; Bento et al., 2010; Klein et al., 2016). However, the full extent to which co-option contributes to the development of novel polyphenisms is not yet known. We show that the response to high temperatures in the mouth-form dimorphism in *P. pacificus* evolved through co-option of a canonical cGMP signaling pathway.

cGMP signaling is present across eukaryotes and has been repeatedly co-opted for a wide range of functions involving the sensing of extracellular environment, including the integration of environmental signals to generate morphology and behaviour, for tissue homeostasis maintenance and as a target for some endotoxins (Sabbatini et al., 2007; Johnson and Leroux, 2010; Rappaport and Waldman, 2018). cGMP-dependent protein kinase *foraging,* which regulates foraging behaviours among insects, has been co-opted in locusts in the regulation of solitary-gregarious transition, and it has more recently been found to regulate caste behaviour regulation in the response to food and temperature in fire ants (Ott et al., 2012; Anreiter and Sokolowski, 2019, Zhou et al., 2020). While we are not aware of previous reports of ion channels in cGMP pathway integration into polyphenisms outside dauer regulation in *C. elegans*, their role in neuronal plasticity has been demonstrated in fruit fly and vertebrates (Zufall et al., 1997; Nishyama et al., 2003; Togashi et al., 2008; Peng et al., 2016; Vonhoff and Keshishian, 2017; Veronica Amarie, 2019).

Thirdly, we show that, while the same pathway is frequently re-used for high temperature sensing by distinct polyphenism among nematodes, its role might drastically differ within similar reaction norms. While co-option re-uses old genetic elements to generate novel phenotypes, developmental system drift invents novel pathways to maintain conserved phenotypes. Based on

morphological segregation in F1 hybrids, DSD has early on been proposed as a candidate. However, only recently it became possible to investigate the non-homologous genetic mechanisms across non-model species, leading to several DSD reports (True and Haag, 2001; Haag, 2014; Nahmad et al., 2008; Lowe and Stolfi, 2018). The mechanisms behind DSD remain unclear.

Evolutionary models showed that DSD may occur when a pleiotropic gene regulates both a phenotype under stabilizing and one under directional selection, such as a novel phenotype (Johnson and Porter, 2007). daf-11 is a pleiotropic gene with functions in salt avoidance, dauer polyphenism and neuron development in C. elegans. Different selective pressures on daf-11 in the evolutionary events which led to P. pacificus, including its novel role in the mouth-form polyphenism, might have led to the change of the regulatory network in dauer formation. Comparative research on molecular mechanisms of C. elegans and P. pacificus dauer larvae have previously suggested DSD in dauer regulation by sensory neurons between the two species. While cumulative loss of sensory cilia in C. elegans leads to dauer constitutive phenotype in C. elegans, it has no effect on dauer formation in P. pacificus (Moreno, Lenuzzi et al., 2018).

We show that DSD and co-option in a temperature-sensing system regulating polyphenism are accompanied by the *cis-* or *trans*-regulatory divergence of the receptor molecule leading to its expression in the different neurons. *daf-11* could have acquired either a novel molecular context within the same neurons, novel functions through heterotopy, or both. Interestingly, as in *C. elegans*' dauer phenotype, AFD, a main thermotactic neuron in *C. elegans*, is probably not the main temperature-sensing neuron in *P. pacificus*' mouth-form dimorphism (Aoki and Mori, 2015; Ramot et al., 2008; Hu, 2018). *However, despite the identification of the homolog of the AFD neuron in P. pacificus,* we cannot exclude that a different neuron altogether is the temperature sensor in *P. pacificus (Hong et al., 2020)*.

Our research paves the way for future investigations of further mechanistic connections between cGMP signaling and downstream master switch molecules in the regulation of *P. pacificus*' polyphenism with the aim of clarifying the complete pathways of signal integration into the switch. It provides a framework for studies of receptive pathways of developmental plasticity in other organisms.

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Supplementary data

Table S1: Statistical comparison of mouth-form phenotype between wild-type RSA635 and mutant lines on 20°C.

line	eu	st	p-value (Fisher's exact test)	FDR	
wild-type	176	72			
daf-25(tu1517)	89	1	3.36E-10	7.05E-09	***
daf-25(tu1516)	70	0	2.02E-09	2.13E-08	***
daf-16(tu1514)	52	63	4.36E-06	3.05E-05	***
daf-16(tu1515)	55	59	5.53E-05	0.000184	***
daf-11(tu1438)	68	18	0.1601584	0.174802	ns

daf-11(tu1439)	78	12	0.002745256	0.004435	***
daf-11(tu1440)	71	19	0.1664776	0.174802	ns
tax-2(tu1291)	57	3	3.63E-05	0.000184	***
tax-2(tu1292)	56	4	0.000178012	0.000312	***
tax-2(tu1296)	42	9	0.1193128	0.139198	ns
RS3410	30	0	9.66E-05	0.000184	***
RS3411	30	0	9.66E-05	0.000184	***
daf-25(tu716)	27	3	0.02859293	0.037528	*
RS3413	30	0	9.66E-05	0.000184	***
RS3414	30	0	9.66E-05	0.000184	***
nag-1(tu719)	30	0	9.66E-05	0.000184	***
RS3416	26	4	0.08290114	0.102407	ns
RS3599	27	3	0.02859293	0.037528	*
daf-11(tu722)	23	7	0.6688908	0.668891	ns
RS3418	28	2	0.007713078	0.01157	*
nag-1(tu724)	30	0	9.66E-05	0.000184	***

Table S2: statistical comparison of mouth-form phenotype between wild-type RSA635 and mutant lines on 27°C.

line	eu	st	p-value (Fishers' exact test)	FDR	
wild-type	43	158			
daf-25(tu1517)	58	7	4.06E-23	7.11E-23	***
daf-25(tu1516)	61	4	9.64E-27	2.02E-26	***
daf-16(tu1514)	10	52	4.69E-01	4.69E-01	ns
daf-16(tu1515)	7	46	0.243825	2.56E-01	ns
daf-11(tu1438)	55	8	2.66E-21	3.99E-21	***
daf-11(tu1439)	56	11	8.33E-20	1.17E-19	***
daf-11(tu1440)	76	14	1.44E-24	2.75E-24	***
tax-2(tu1291)	37	5	3.11E-16	3.84E-16	***
tax-2(tu1292)	47	8	2.81E-18	3.69E-18	***
tax-2(tu1296)	6	4	0.011659	1.29E-02	*
RS3410	37	6	1.51E-15	1.76E-15	***
RS3411	99	0	2.31E-45	2.43E-44	***
daf-25(tu716)	127	1	4.57E-52	9.59E-51	***

RS3413	98	16	3.73E-30	8.69E-30	***
RS3414	75	18	2.46E-22	3.98E-22	***
nag-1(tu719)	69	0	7.78E-35	2.33E-34	***
RS3416	102	5	1.11E-39	4.65E-39	***
RS3599	94	1	5.17E-42	2.71E-41	***
daf-11(tu722)	108	7	2.26E-39	7.91E-39	***
RS3418	80	6	1.09E-31	2.85E-31	***
nag-1(tu724)	96	0	2.20E-44	1.54E-43	***

Table S3: within line statistical comparison of mouth-form phenotype.

line.temperature	**eu**	**st**	**p-value (Fisher's exact test)**	**FDR**	
wild-type.20	176	72			
wild-type.27	43	158	1.67E-26	1.83E-25	***
daf-25(tu1517).20	89	1			
daf-25(tu1517).27	58	7	9.85E-03	2.71E-02	*
daf-25(tu1516).20	70	0			

daf-25(tu1516).27	61	4	5.12E-02	1.13E-01	ns
daf-16(tu1514).20	52	63			
daf-16(tu1514).27	10	52	1.21E-04	4.45E-04	***
daf-16(tu1515).20	55	59			
daf-16(tu1515).27	7	46	1.17E-05	6.46E-05	***
daf-11(tu1438).20	68	18			
daf-11(tu1438).27	55	8	2.75E-01	3.36E-01	ns
daf-11(tu1439).20	78	12			
daf-11(tu1439).27	56	11	6.51E-01	6.51E-01	ns
daf-11(tu1440).20	71	19			
daf-11(tu1440).27	76	14	4.41E-01	4.85E-01	ns
tax-2(tu1291).20	57	3			
tax-2(tu1291).27	37	5	2.69E-01	3.36E-01	ns
tax-2(tu1292).20	56	4			
tax-2(tu1292).27	47	8	2.26E-01	3.36E-01	ns
tax-2(tu1296).20	42	9			

tax-2(tu1296).27	6	4	1.98E-01	3.36E-01	ns

Table S4: statistical comparison of dauer numbers between wild-type RSA635 and *tu1440* and RSA635 wild-type and *tu1296* at 20°C.

line	non-dauer	dauer	p-value (Fisher's exact test)	
RSA635	393	158		
daf-11(tu1440)	419	77	3.94E-07	***
tax-2(tu1296)	417	1	2.74E-42	***

Table S5: statistical comparison of dauer numbers between wild-type RSA635 and *tu1440* and RSA635 wild-type and *tu1296* at 27°C.

line	non-dauer	dauer	p-value (Fisher's exact test)	
RSA635	321	258		
daf-11(tu1440)	483	39	2.31E-47	***
tax-2(tu1296)	433	0	2.77E-77	***

Table S6: within line statistical comparison of dauer numbers between 20° and 27°C

line.temp	non-dauer	dauer	p-value (Fisher's exact test)	
RSA635.20	393	158		
RSA635.27	321	258	3.67E-08	***
daf-11(tu1440).20	419	77		

daf-11(tu1440).27	483	39	6.88E-05	***
tax-2(tu1296).20	417	1		
tax-2(tu1296).27	433	0	4.91E-01	ns

Table S7: Phenotype of wild-type RSA635 on different concentrations of NaCl in NGM agar. For "no salts" treatment, NaCl, KPO4 and MgSO4 were excluded from the standard NGM formula. In all other experiments, standard NGM formula was used with altering levels of NaCl (n=3).

treatment	eu	st	% eu
no salts (-K2HPO4, K2SO4, NaCl)	19	33	36.53846
0mM NaCl	59	1	98.33333
50mMNaCl (standard NGM)	256	28	90.14085
100mM NaCl	12	24	33.33333
200mM NaCl	6	29	17.14286