Modes of Inheritance and Adaptive Values of Mouth-Form Dimorphism in the Nematode *Pristionchus pacificus*

Dissertation
der Mathemathisch-Naturwissenschaftlichen Fakultät
der EBERHARD KARLS UNIVERSITÄT TÜBINGEN
zur Erlangung des Grades eines
Doktors der Naturwissenschaften
(Dr. rer. nat.)

vorgelegt von
Vahan Serobyan
aus Vanadzor, Armenia

Tübingen
2015

Gedruckt mit Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät der EBERHARD KARLS UNIVERSITÄT TÜBINGEN.

Tag der mündlichen Qualifikation:

Dekan: Prof. Dr. Wolfgang Rosenstiel

1. Berichterstatter: Prof. Dr. Ralf J. Sommer

2. Berichterstatter: Prof. Dr. Alfred Nordheim

Contents

Summary 6

Zusammenfassung 7

List of Publications 8

1 Introduction 9

1.1 Evolutionary developmental biology 9

1.2 Phenotypic plasticity 11
1.2.1 Polyphenism as a special case of phenotypic plasticity 12
1.2.2 Developmental switches 12
1.2.3 Phenotypic plasticity and epigenetic 13
1.2.3.1 DNA methylation 15
1.2.3.2 Chromatin Modifications 16
1.2.3.3 Alternative splicing 18
1.2.3.4 Non-coding RNAs 18
1.2.4 Phenotypic plasticity and maternal effects 21

1.3 *Pristionchus pacificus* from a satellite model organism to a model organism 24

1.4 *P. pacificus* mouth dimorphism as an example of phenotypic plasticity 27

1.5 Nematodes as a model for studying epigenetic mechanisms and developmental plasticity 30

1.6 Aim of the thesis 31

2 Results and Discussion 32

2.1 Feeding plasticity in the nematode *Pristionchus pacificus* is influenced by sex and social context and is linked to developmental speed 32
2.1.1 Synopsis 32
2.1.2 Own Contribution 33

2.2 Adaptive value of a predatory mouth-form in a dimorphic nematode 34
2.2.1 Synopsis 34
2.2.2 Own Contribution 35

2.3 Chromatin remodeling and antisense-mediated up-regulation of the developmental switch gene *eud-1* control a predatory feeding decision 36

2.3.1 Synopsis 36
2.3.2 Own Contribution 37

2.4 Mapping, cloning and characterization of genes regulating *P. pacificus* mouth-form dimorphism in males 38
2.4.1 Introduction 38
2.4.2 Genetic screen for maternal-infuence defective phenotype 38
2.4.3 *mid-1* encodes a SR proteine kinase 39
2.4.4 *mid-2* might encode a lncRNA 43
2.4.5 Material and methods 44

References 48

Acknowledgements 71

Curriculum Vitae 73

Appendix 75

Summary

Developmental (phenotypic) plasticity is the ability of an organism to change its phenotype in response to altered environmental conditions. Polyphenisms are a special case of developmental plasticity, leading to alternative phenotypes that can be functionally independent and result from developmental switches. The nematode *Pristionchus pacificus*, a laboratory model for comparative mechanistic biology, shows phenotypic plasticity in its feeding structures and has emerged as a model for investigating the interface between regulatory developmental mechanisms and interactions with the environment. *P. pacificus* shows a dimorphism of its mouthparts and animals are either "stenostomatous" (St) with a narrow mouth or "eurystomatous" (Eu) with a wide mouth. Recent studies have shown that the irreversible developmental switch between these two forms involves the sulfatase *eud-1*, which also controls the micro- and macroevolution of the mouth-form ratio in *Pristionchus* species.

The primary aim of this thesis is to contribute to a systematic genetic and molecular characterization of mouth-form plasticity in *P. pacificus*. I show that plasticity is sex-linked and that there is a correlation between the mothers' mouth-form and that of her male progeny. Moreover, I show that the two forms have conditional fitness advantages in different environments; St worms develop faster on bacterial diet, whereas Eu animals have an advantage as predators when only a prey diet is provided.

To identify the genetic basis and molecular mechanisms controlling the developmental switch, I performed a mutagenesis screen and additionally screened the *P. pacificus* mutant collection for pleiotropic mutants. Analyzing mutations at five different loci I show that mouth-form plasticity is under strong epigenetic control, including chromatin remodeling and long non-coding RNA-mediated gene regulation. Most importantly, I identified a novel antisense transcript at the *eud-1* locus, which acts as a positive regulator of the sulfatase-encoding *eud-1* transcript. Together, this study provides first inroads into the epigenetic control of *P. pacificus* mouth-form plasticity and identifies an unexpected level of complexity in the regulation of a simple dimorphism.

Zusammenfassung

Phänotypische Plastizität (Entwicklungsplastizität) ist die Fähigkeit eines Organismus seinen Phänotyp als Reaktion auf wechselnde Umweltbedingungen zu verändern. Polyphänismen sind ein besonderer Fall der phänotypischen Plastizität, die zu alternativen Phänotypen führen, die funktionell unabhängig sein können und auf Entwicklungsschalter zurückzuführen sind. Der Nematode *Pristionchus pacificus*, ein Labormodell für die vergleichende mechanistische Biologie, zeigt eine phänotypische Plastizität seiner Mundöffnung und wurde bekannt als Modell, um die Verbindung zwischen regulatorischen Entwicklungsmechanismen und Umweltinteraktionen zu erforschen. *P. pacificus* weist einen Dimorphismus seiner Mundform auf und die Tiere sind entweder "stenostomat" (St) mit einer schmalen Mundöffnung oder "eurystomat" (Eu) mit einer breiten Mundöffnung. Neuste Untersuchungen zeigten, dass die Sulfatase *eud-1* sowohl die irreversible Umschaltung zwischen diesen beiden Formen als auch die Mikro- und Makroevolution des Mundformverhältnisses in *Pristionchus* Arten steuert.

Das Hauptziel dieser Arbeit ist zu einer systematischen genetischen und molekularen Charakterisierung der Mundformplastizität in *P. pacificus* beizutragen. Ich zeige, dass diese Plastizität geschlechtsspezifisch ist und dass eine Korrelation zwischen der Mundform der Mutter und die ihrer männlichen Nachkommen besteht. Außerdem zeige ich, dass die zwei Formen gewisse Fitnessvorteile unter verschiedenen Umweltbedingungen haben; St Würmer entwickeln sich schneller bei bakterieller Ernährung, wohingegen Eu Tiere einen Vorteil als Räuber haben, wenn nur Beutetiere als Nahrungsquelle bereitgestellt werden.

Um die genetische Grundlage und die molekularen Mechanismen zur Steuerung des Entwicklungsschalters zu bestimmen, führte ich ein Mutagenese-Experiment durch und durchsuchte zusätzlich die *P. pacificus* Mutantensammlung nach pleiotropischen Mutanten. Indem ich Mutationen in fünf verschiedenen Loci analysierte, konnte ich zeigen, dass die Mundformplastizität unter starker epigenetischer Kontrolle steht, einschließlich Chromatin-Remodeling und lange nichtcodierende RNA-vermittelte Genregulation. Mein wichtigstes Ergebnis war die Entdeckung eines neuen antisense Transkripts im *eud-1* Locus, welches als positiver Regulator des die Sulfatase codierenden *eud-1* Transkripts fungiert. Diese Arbeit liefert erste Einblicke in die epigenetische Kontrolle der *P. pacificus* Mundformplastizität und zeigt ein unerwartet hohes Maß an Komplexität in der Regulation eines einfachen Dimorphismus.

List of Publications

Serobyan V., Ragsdale E. J., Müller M. R. and Sommer R. J. (2013). Feeding plasticity in the nematode *Pristionchus pacificus* is influenced by sex and social context and is linked to developmental speed. Evolution & Development 15: 161-170.

Serobyan V., Ragsdale E. J. and Sommer R. J. (2014). Adaptive value of a predatory mouth-form in a dimorphic nematode. *Proceedings of the Royal Society of London B* 281: 20141334

Serobyan V., Xiao H., Rödelsperger C., Namdeo S., Röseler W., Witte H. and Sommer R. J. (submitted 2015) Chromatin remodeling and antisense-mediated up-regulation of the developmental switch gene *eud-1* control a predatory feeding decision

1 Introduction

1.1 Evolutionary developmental biology

Evolutionary developmental biology (evo–devo) investigates the mechanistic relationships between the processes of individual development and phenotypic change during the course of evolution. Hence, evo-devo is the merger of two disciplines: evolutionary biology and developmental biology (Raff, 1996;Wilkins, 2002; Arthur, 2002; Sommer, 2009). Evo–devo portrays a mechanistic approach for the understanding of phenotypic change in evolution. In this, it differs substantially from the mainstream theory of evolution, which is based on the correlation of phenotypic variation with statistical gene frequencies in populations (Arthur, 2002; Davidson and Erwin, 2006; Sommer, 2009).

Evo-devo also differs conceptually from mainstream evolutionary biology. For example, the explanation of adaptive change as a population-dynamic event is the central goal of the modern synthesis of evolution. In contrast, evo-devo aims to understand the developmental processes by which an organism grows from a single fertilized egg to the adult stage in an evolutionary context (Wilkins 2002). The construction of the embryo is a complex process, including cell proliferation, cell specialization, cell interactions and cell movement. All these processes are controlled by differential gene expression. The complexity of an organism depends on the remarkable feature of the genetic regulatory system. However, the expression of genes depends not only on the genetic material inherited from parental cells but also on the cells' present and past environments. Hence, any phenotype is a product of both, genotype and environment, and the interaction of both (Arthur, 2002; Davidson and Erwin, 2006; Gilbert and Epel, 2009).

Evo–devo attempts to expound phenotypic change through the variations in developmental mechanisms (the physical interactions among genes, cells and tissues, as well as cell-environmental interactions), and whether they are adaptive or not. This addresses many of the principal features of phenotypic

change, *e. g.* the generation of new structural elements (phenotypic novelty), the establishment of standardized building units (modularity, homology), the arrangement of such units in lineage-specific combinations (body plans), and the repeated generation of similar forms in independent taxa (homoplasy) (Raff and Wray, 1989; von Dassow and Munro, 1999; West-Eberhard, 2003). Also, evo–devo aims to explain the evolution of development itself and how the control of developmental processes is accomplished by the interplay between genetic, epigenetic and environmental factors. With these goals, evo–devo moves the focus to the qualitative phenomena of phenotypic organization and their mechanistic causes. The major deviations of evo–devo from the standard theory of evolution are characterized by the terms evolvability, emergence and organization (Nylin and Gotthard, 1998).

The most prominent issue of evo-devo is the origin of phenotypic organization: how certain constructional motifs arise, how they become conserved and integrated into the body architecture, and how they are reused over and over again. One striking feature observed in several evo-devo studies is that phenotypic architecture is more robust than many of the suites of molecular and developmental interactions that are involved in its formation. This originally surprising finding has been called 'developmental systems drift' and is now developing into a truism of molecular biology and evolution (True and Haag, 2001). This includes many more factors than the evolution of gene regulatory networks alone, notably the dynamics of epigenetic interactions, the chemical and physical properties of growing cell and tissues, and the influences of environmental parameters (Arthur, 2000; 2002; Jablonka and Lamb, 2005; Sommer, 2009).

1.2 Phenotypic plasticity

Phenotypic plasticity is the capacity of a single genotype to produce different phenotypes in response to changing external conditions (West-Eberhard, 2003). Starting from the zygote all developmental programs are originally plastic. The phenotypic outcome will change depending on environmental conditions, physical and chemical interactions, and genetic and epigenetic changes (West-Eberhard, 2003). For example, experiments of developmental biology pioneers demonstrated that cell-cell interactions are important for embryonic development. If one of the blastomers of a developing amphibian embryo is burned but not removed, only half of the embryo will develop. However, if blastomers are physically separated, they can develop as identical twins (Spemann, 1938). Decades later, it was shown that these cell-cell interactions are not only physically important but that there are secreted proteins regulating the normal development of the embryo (De Robertis, 2006).

Although, by default, many developmental programs are plastic, the biological robustness we see is a result of evolution. Such robustness of developmental pathways shaped by evolution has been called ‘canalization’ or ‘stabilizing selection’ (Waddington, 1942; Schmalhausen, 1949). Vulva development in the nematode *Caenorhabditis elegans* is an interesting example, illustrating how the expression of genetic variation can be canalized in a way that several genotypes produce similar, selectively equivalent phenotypes (Fèlix and Wagner, 2008).

In a constantly changing environment, plasticity can be beneficial (Liefting *et al.*, 2009). Hence, phenotypic plasticity is one of the salient themes in evo–devo. The role of plasticity in evolution can be measured by the slope of 'developmental reaction norms' (de Jong, 1990; Pigliucci *et al.*, 1996); *i. e.*, functions that relate the response of a genotype to a specific environmental perturbation. Plasticity is often discussed to be adaptive because it results in increased fitness of the organism under particular circumstances compared to organisms that are not plastic. However, plasticity can also be non-adaptive, if it

results from a passive response to environmental conditions (Price *et al.*, 2003; Liefting *et al.*, 2009; Schlichting and Wund, 2013). The level of plasticity is probably under selection and depends on environmental conditions (changing *vs.* stable environment) (Via and Lande, 1985).

1.2.1 Polyphenism as a special case of phenotypic plasticity

A special case of phenotypic plasticity is polyphenism, when environmental conditions can induce two or more discrete alternative phenotypes. Thus, polyphenisms are examples where phenotypic plasticity produces two or more alternative phenotypes in a genetically identical background. The mediating role of the environment is important under such circumstances (Abouheif and Wray, 2002; Nijhout, 2003, Suzuki and Nijhout, 2006). Specifically, developmental outcome can be controlled by (1) endocrine and hormone activity, for example (1a) social caste in ants (Abhouef and Wray, 2002), (1b) honeybees, termites and aphid wing development (Miura, 2005), (2) seasonal factors (Brakefield, 1991; Roskam and Brakefield, 1999), (3) predator-induction (Stibor, 1992; Tollrian and Harvell, 1999), and similarly, (4) nutrient availability (Pfennig, 1990; Newlon *et al.*, 2003), (5) environmental regulation (Nijhout, 1999) and (6) presence of conspecifics and pheromones (Fielenbach and Antebi, 2008; Bose *et al.*, 2012).

1.2.2 Developmental switches

Theoretical and empirical geneticists have long acknowledged that the existence of developmental switch mechanisms is essential for the irreversible control of plasticity (Waddington 1953; Mather, 1953; 1973; Scharloo, 1970). Thoday's experiments, using disruptive selection for the extremes of continuously variable traits, dissected the molecular basis of developmental switch mechanisms (Thoday, 1964). This was the first empirical work demonstrating the origin of an environmentally induced polyphenism under disruptive selection.

Recent advances in genome-wide identification of target genes allow researcher identify genes controlling switches of developmental programs (Golden and Riddle, 1984; Kaufman *et al*., 2010; Rodenas-Ruano *et al*., 2012; Ragsdale *et al*., 2013a). An interesting example of the analysis of a developmental switch mechanism was provided by the cloning of the *eud-1* gene in the nematode *Pristionchus pacificus* (Ragsdale *et al*., 2013a). Ragsdale and colleagues (2013a) demonstrated that loss-of-function alleles of a single gene can fix one of the alternative forms of a polyphenism, while the overexpression fixes the other form. Interestingly, the same gene is also involved in regulating this polyphenism at the macroevolutionary and microevolutionary levels and will be discussed in more details below [see 1.4].

Despite recent interests in switch mechanisms controlling plasticity, it is still not well-understood how developmental switch mechanisms act at the molecular level and how they can switch an entire developmental program that often includes hundreds of genes. In some studies it has been demonstrated that these genes are often subject to epigenetic regulation (Uchida *et al*., 2010; Johnson and Spence, 2011; Rodenas-Ruano *et al*., 2012), but even in these cases many details remain unclear.

1.2.3 Phenotypic plasticity and epigenetics

The original definition of epigenetics was by Waddington in 1942. According to this definition the phenotype emanates from the genotype via programmed change (Van Speybroeck, 2002). "An epigenetic trait is a stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence." (Reviewed in Berger *et al*., 2009). Similarly, phenotypic plasticity is defined as the ability of a genotype to develop alternative phenotypes in response to environmental change without changes in DNA (Kelly *et al*., 2012). In other words, plasticity and epigenetic modification share the distinction of causing changes in phenotype or gene expression without changes in DNA sequence. Also, the phenotypic expression of plasticity is a response to the

environment. Many epigenetic marks are environmentally induced and plasticity itself can be mediated by epigenetic modification, and transgenerational (parental) effects, may be due to maternal epigenetic inheritance.

The evolutionary importance of epigenetic marks has been valued by many researchers (Jablonka and Lamb,1998 1999 2005; Kalisz and Purugganan, 2004; Rapp and Wendel, 2005; Wolf and Hager, 2006; Bonduriansky and Day, 2009; Gilbert and Epel, 2009; Feinberg and Irizarry, 2010; Johnson and Tricker, 2010; Richards *et al.*, 2010; Shea *et al.*, 2011; Dickins and Rahman, 2012; Jablonka, 2013; Smith and Ritchie, 2013). Although, there is sufficient data suggesting that the inheritance of epigenetic marks is extremely common (Jablonka and Raz, 2009) and transgenerational plasticity has been increasingly entrained in adaptation (Becker *et al.*, 2011; Schmitz *et al.*, 2011), experimental studies investigating the evolutionary role and transgenerational effects are still in their infancy.

The role of epigenetic regulation of plastic development is often discussed in human disease biology. For example, Feinberg characterizes disease epigenetics as defects in phenotypic plasticity (Feinberg, 2007). Indeed, it has become increasingly clear that 1) DNA methylation (Feinberg and Vogelstein, 1983; Wu *et al.*, 2005), 2) histone modification (Varambally *et al.*, 2002; Bachman, *et al.*, 2003; Fraga, *et al.*. 2005) 3) microRNAs and 4) other noncoding RNAs (Li *et al.*, 2015; Kan *et al.*, 2015; Li *et al.*, 2015; He and Wang, 2015) are altered in cancer, given their interdependence in normal development (Feinberg and Tycko, 2004; Jones and Baylin, 2002; Feinberg, 2007). However, many of these epigenetic regulators are key players not only in cancer, but also in other human diseases, as they are regulators of gene expression during development and are frequently mis-expressed in human disease states (Amir *et al.*, 1999, Shahbazian *et al.*, 2002; He *at al.*, 2011; Qureshi and Mehler, 2014; Tushir and Akbarian, 2014; Hoss *et al.*, 2014; Brookes *et al.*, 2015).

Therefore, epigenetics offers an important paradigm to understand the role of the environment's interactions with the genome. During the development of an organism, cells are able to change their behaviour in response to internal and

external environmental factors (Bjornsson *et al*., 2004). Although such effects ultimately feed into developmental genetic pathways (Lehner *et al.*, 2006), the actual phenotypic change depends on epigenetic regulations that are mediated by factors including diet, pH, humidity, temperature, photoperiod, seasonality, population density or the presence of predators. In the following, I provide a short overview on the different molecular mechanisms that have been identified to be involved in epigenetic regulations.

1.2.3.1. DNA methylation

DNA methylation is the addition of methyl groups to the DNA molecule (Riggs, 1975; Noyer-Weidner and Trautner, 1993; Bird *et al*., 1995). DNA methylation occurs right after DNA replication by DNA methyltransferases (Bestor and Ingram, 1983). This methyl group may be added to the fifth or fourth carbon atom of the cytosine base or the nitrogen atom N6 of the adenine base (Dodge *et al*., 2002; Suzuki and Bird 2008; Iyer *et al*., 2011; Greer *et al*., 2015). Such DNA modifications can change gene expression in cells when they divide and differentiate from embryonic stem cells into cells of a particular tissue. The process of DNA methylation is stopped when a zygote is formed but is restored as cell division occurs during development (Jaenisch and Bird, 2003; Qiu, 2006; Bell and Felsenfeld, 2000; Chodavarapu *et al*., 2010). It was shown that targeted mutations of DNA methyltransferases result in embryonic lethality (Li *et al*., 1992).

One essential function of DNA methylation is the formation of chromatin structure and chromosome stability (Buschausen et al., 1987; Cooper and Youssoufian, 1988; Bird and Wolffe, 1999). Also, DNA methylation is significant for normal development and is linked to several processes such as genomic imprinting, oncogenesis and the suppression of repetitive elements (Cattanach and Kirk, 1985; Zhang *et al*., 2011; Yu *et al*., 2014). DNA methylation also enables the expression of retroviral genes to be suppressed, along with other potentially dangerous sequences of DNA that have entered and may damage the

host (Nartey *et al.*, 2014; Yu *et al.*, 2014). Similarly, DNA methylation has a vital role in silencing the transcription of retrotransposones during embryonic development and spermatogenesis (Walsh et al., 1998; Bourc'his and Bestor 2004).

Additionally, DNA methylation is important for the development of cancer and is a key regulator of gene transcription (Egger *et al.*, 2004). Multiple studies have shown that genes with a promoter region that contains a high concentration of 5-methylcytosine are transcriptionally silent (Wolf *et al.*, 1984; Robertson *et al.*, 1996; Mani and Herceg, 2011). DNA hypermethylation is linked to the activation of genes and DNA hypomethylation has been described to be associated with the development of cancer through various mechanisms (Baylin *et al.*, 1987; Pelch *et al.*, 2015).

1.2.3.2 Chromatin Modifications

Chromatin is the complex unit of DNA and five highly conserved proteins, the histones (Kornberg, 1977). Four of these proteins, H2A, H2B, H3 and H4, make dimers that are assembled into an octamer that has 145–147 base pairs (bp) of DNA wrapped around it to form a nucleosome core (Luger *et al.*, 1997), which later assembles with histone H1 (Ramakrishnan *et al.*, 1993). The linker histone H1 does not form part of the nucleosome, but acts as a stabilizer of the internucleosome DNA. H1 histones are important regulators of the structural and functional properties of the dynamic chromatin fiber (Misteli *et al.*, 2000; Bustin *et al.*, 2005). The functions of chromatin are to condense DNA into smaller volume and strengthen the DNA to allow mitosis and control gene expression and DNA replication (Bannister and Kouzarides, 2011).

Modifications of histone N-terminal tails can effect inter-nucleosomal interactions and thus affect overall chromatin structure, transcription and many other DNA processes such as repair, replication and recombination (Luger *et al.*, 1997; Muegge, 2003; Bannister and Kouzarides, 2011; Maile *et al.*, 2015). Based on this modification and their combinations chromatin has two different

states (1) euchromatin, where DNA is accessible for transcription, and (2) heterochromatin, where DNA is more compact and therefore less accessible for transcription (Muegge, 2003; Grewal and Jia, 2007; Roudier *et al.*, 2011). By now, there are many types of histone modifications described by researchers. Among these (1) acetylation, methylation and ubiquitination of lysine, (2) methylation of arginine, and (3) phosphorylation of serine, threonine and tyrosine are the most common modifications (Sanders *et al*, 2004; Tjeertes *et al.*, 2009; Bannister and Kouzarides, 2011; Zentner and Henikoff, 2013; Basnet *et al.*, 2014; Nguyen *et al.*, 2014; Hu *et al.*, 2014; Wright and Kao, 2015; Mitchell *et al.*, 2015, Noh *et* al., 2015).

There are strong correlations between different histone marks, which are often called cross-talk of histone marks (Lee and Shilatifard, 2007; Duncan *et al.*, 2008; Li *et al.*, 2008; Whitcomb *et al.*, 2012; Schwammle *et al.*, 2014; Cole *et al.*, 2015). A well-studied example of such a cross-talk is monoubiquitination of H2B (H2Bub), which stimulates trimethylation of histone H3 Lys4 (H3K4me3), although trimethylation of H3K4 mark can happen independently from H2Bub (Whitcomb *et al.*, 2012; Wu *et al.*, 2013; Thornton *et al.*, 2014). A similar cross-talk was observed between histone marks H2Bub an H3K79me (Lee *at al.*, 2007). Another example of histone cross-talk is phosphorylation of H3S10 which promotes the acetylation of H3K4 site in yeast (Daujat *et al.*, 2002). These examples indicate that the histone code is a complex 'language' with diverse functional consequences (Berger and Shelley, 2007; Suganuma and Workman, 2008). Although results in global epigenetic projects have huge impacts on systems biology (Cusanovich *et al.*, 2015; Maile *et al.*, 2015), there is still lack of basic knowledge on histone modifications and their effects on individual gene behaviour.

1.2.3.3 Alternative splicing

Living organisms are able to generate all necessary proteins from a relatively small number of genes (Nilsen and Graveley, 2010). Alternative splicing is a mechanism that increases the coding potential of the genome (Zhou *et al.*, 2014).

During alternative splicing from coding gene some exons might be included, whereas some others excluded in a way that mature messenger RNA (mRNA) differs in their encoding aminoacid sequence. Hence different isoforms of a gene might have different functions (Black, 2003; David and Manley, 2008).

Genome wide analyses show that up to 95% of the genes in the human genome have multiple exons undergoing alternative splicing (Pan *et al.*, 2008, Castle *et al.*, 2008; Lee and Rio, 2015). Alternative splicing is often regulated by local histone modifications (reviewed in Zhou *et al.*, 2014). The analyses of genome-wide chromatin immunoprecipitation-sequencing (ChIP-seq) data suggest that there is enrichment of H3K36me3 marks on exons, and this histone mark is strongly associated with constitutive exons than with alternative exons (Spies *et al.*, 2009; Schwartz *et al.*, 2009; Kolasinska-Zwierz *et al.*, 2009). Also, there were reports for other histone marks, including H3K4me1, HJ3K27me2, H3K4me3, H4K20me1, H2B3K5me1, H3K79me1, that have enrichment correlation with exons, a phenomenon conserved among metazoans (Spies *et al.*, 2009; Schwartz *et al.*, 2009; Anderson *et al.*, 2009; Kolasinska-Zwierz *et al.*, 2009; Pradeepa *et al.*, 2014). A similar evolutionary conserved mark of exon definition is DNA methylation (Hodges *et al.*, 2009; Chodavarapu *et al.*, 2010).

1.2.3.4 Non-coding RNAs

Non-coding RNAs (ncRNAs) are functional RNA molecules that are transcribed from DNA but are not translated into proteins (Mattick and Makunin, 2006; Kanhere and Jenner, 2012). Generally, the function of ncRNAs is to regulate gene expression at the transcriptional and post-transcriptional level

(Kapranov et al., 2007; Mercer et al., 2009; Zhou *et al.*, 2014). Until recently the common idea about the functions of RNA was that they are informational intermediates between DNA and encoded protein (Mattick and Makunin, 2006). Indeed, most known RNAs had very generic function in cells, including mRNA translation (tRNAs and rRNAs), splicing (small nuclear RNAs (snRNA)) and modification of rRNAS (smallnucleolar RNAs (snoRNA)) (Mattick and Makunin, 2006; Kishore and Stamm, 2006; Zhou et al., 2014). It is now known that RNAs are involved not only in protein synthesis but also in DNA replication, post-transcriptional modification in regulatory mechanisms, including transcriptional attenuation, mRNA degradation and stabilization, and gene regulation (Pannucci *et al.*, 1999; Brantl, 2007; Lin *et al.*, 2006; Ahmad and Henikoff, 2002; Perreault *et al.*, 2007; Kota *et al.*, 2015).

ncRNAs that are involved in gene regulation mechanisms can be classified into two major groups: the short ncRNAs (<30 nts) and the long ncRNAs (>200 nts) (Moazed, 2009; Jung *et al.*, 2010). The three major classes of short non-coding RNAs are microRNAs (miRNAs), short interfering RNAs (siRNAs), and piwi-interacting RNAs (piRNAs) (Dozmorov *et al.*, 2013). They are not discussed here in more deteil because they are not relevant for the work to be described below.

Long nc (lnc) RNAs are more then 200 nucleotide long RNA molecules that do not have an open reading frame and are often polyadenylated (Derrien *et al.*, 2012; Batista and Chang, 2013). Many of the known lncRNAs make ribonucleoprotein complexes that influence different stages of gene expression (Rinn and Chang, 2012; Fatica and Bozzoni, 2014; Holoch and Moazed, 2015). Also, they can make complexes with chromatin modifying proteins and regulate gene expression (Mercer and Mattick, 2013; Holoch and Moazed, 2015).

lncRNAs are known to be involved in chromatin remodeling, transcriptional regulation, post-transcriptional regulation and as a precursor of siRNAs (Allison *et al.*, 2014; Holoch and Moazed, 2015). Conventionally, lncRNAs are divided in two groups: lncRNAs that act in the nucleus and those that act in the cytoplasm (Khalil *et al.*, 2009; Batista and Chang, 2012; Fatica and Bozzoni, 2014).

lncRNAs can act in *cis* and *trans*, and they can regulate gene expression negatively or sometimes positively and have direct or indirect effects (Ørom *et al*., 2010; Salmena *et al*., 2011; Yoon *et al*., 2012; Carrieri *et al*., 2012; Rinn and Chang, 2012; Batista and Chang, 2013; Guttman and Rinn, 2012; Wang *et al*., 2014; Marquardt *et al*., 2014; Barriocanal *et al*., 2015).

A widely known and best-characterized lncRNA is the X-inactive specific transcript gene (*Xist*). This gene has an important role in dosage compensation, specifically X-chromosome inactivation in mammals. This process involves two ncRNAs; *Xist* and its antisense transcript *Tsix*, a negative regulator of *Xist*. Before differentiation, both, *Xist* and *Tsix* are actively transcribed due to H3K4 dimethylation of the *Xist* gene. In this state X-chromosome inactivation is a random process. Upon differentiation, *Xist* expression is elevated resulting in *Xist* RNA coating the future inactive X chromosome, which triggers extensive histone methylation and X chromosome inactivation. This process balances the difference in X-chromosome dosage between two sexes (Lee *et al*., 2000; Sado *et al*., 2001; Schoeftner *et al*., 2006; Lee and Bartolomei, 2013; Nozawa *et al*., 2013). The deletion of *Xist* in mice causes X-chromosome inactivation and female-specific lethality (Penny *et al*., 1996). *Xist* is controlled by two RNA-based switches: *Tsix* for the active chromosome X and *Jpx* (X inactive chromosome encoded activator) for the inactive chromosome X (Tian *et al*., 2010).

Another example of well-studied lncRNA is *MALAT1*, a transcript that was originally identified as an abundantly expressed ncRNA that is upregulated during metastasis of early-stage lung cancer. The expression level of *MALAT1* is used for diagnostic purposes of lung cancer (Fu *et al*., 2006). *MALAT1* acts in nucleus and the loss-of-function mutations in embryos result in normal development (Lai *et al*., 2012).

Another interesting group of lncRNAS that are associated with chromatin modifying complexes are intergenic non-coding RNAs (lincRNAs). lincRNAs can target specific genomic loci to promote specific epigenetic states (Fatica and Bozzoni, 2014; Kannan *et al*., 2015). Similarly, circular RNAs (circRNAs) in animals are an interesting class of RNA. circRNAs can be abounded and some of

them are conserved across different taxa. This suggests evolutionary importance for the role of this RNA class, however, the specific function remains currently unknown (Jeck *et al*., 2013; Memczak *et al*., 2013). circRNAs got their name, because 3' and 5' ends of the RNA molecule are joined together (Salzman *et al*., 2012; Guo *et al*., 2014). cirRNAs are mainly found in the cytoplasm. However, it is not known how these molecules exit from the relatively small nuclear pore (Fatica and Bozzoni, 2014). One hypothesis is that the exit of these cirRNAs occurs during mitosis, when the nuclear envelope breaks down (Jeck *et al*., 2013). Interestingly, cirRNAs are found also in neurons where mitotic division is not frequent (Memczak *et al*., 2013).

Taken together, the molecular mechanisms through which epigenetic factors regulate gene expression are now understood in a great detail. These factors are often associated with transgenerational effects and might have an evolutionary importance because they are necessary keys of developmental programs (Rodenas-Ruano *et al*., 2012; van Heesbeen *et al*., 2013; Resendiz *et al*., 2013).

1.2.4 Phenotypic plasticity and maternal effects

The phenotype of an organism is determined not only by the genotype and the environment, but also by the environment and genotype of its mother (Nussey *et al.*, 2007). So-called maternal effects are common in animals because mothers deliver some mRNAs or proteins to the oocyte. These mRNAs are essential for the embryo, because in many organisms during early embryogenesis the embryo is transcriptionally inactive (Schier, 2007). However, later this maternal control of development is gradually replaced by embryonic control (Labouesse and Mango, 1999). Due to the fact that the phenotype of an offspring is determined by the mother's genotype, the genetic screens for the identification of maternally required genes have proven to be difficult for geneticists (Jorgensen and Mango, 2002). Maternally required genes have been studied systematically in *Drosophila melanogaster* segmentation, and many

maternal effect mutants have been generated that are affected at the early stages of embryogenesis (Nüsslein-Volhard *et al*., 1980; Schüpbach and Wieschaus, 1986; Nüsslein-Volhard *et al*., 1987).

However, maternal effects are not only limited by transferring mRNA or proteins that are required for early embryogenesis before the embryos themselves are transcriptionally active. They also become apparent in maternal photoperiod, maternal diet, the temperature of chosen nest or secondary sexual characters of mating partner and in case of parasites and parasitoids, or host choice (Bernardo, 1996; Fox and Mousseau, 1998; Mousseau and Fox, 1998, Nussey *et al*., 2007). Both, maternal environment and behaviour can influence offspring phenotype. Therefore, it is most likely that maternal effects are under the pressure of natural selection.

Maternal effects can in theory provide a mechanism for adaptive transgenerational phenotypic plasticity, in which the environment experienced by the mother is translated into phenotypic variation in the offspring, and this relationship can be visualized as a reaction norm (Mousseau and Fox, 1998). Also, in this case, plasticity results in alternative phenotypes that are developmentally and functionally different and independent of each other. Hence they can be independently subjected to selection and evolution (Nijhout, 2003). Based on theoretical and experimental studies, it has been suggested that maternal effects have a significant impact on evolution. However these predictions suppose that maternal effects themselves are evolutionarily constant (Galloway, 2005).

In summary, developmental plasticity is an important theme in evo–devo because it elucidates to the relationships among trait variation, natural selection and environmental influence. Plasticity suggests that selection can regulate all stages of an organisms' development, and it provides a key to understand the mechanisms involved in the regulating of populations under changing environmental conditions. Studies of environment-dependent trait correlations and plastic responses across different environments show that changing conditions can be met with coordinated reactions. Selection might favour

developmental systems that actually reduce integration, in order to allow adjustments of the interactions among traits as a response to environmental conditions (Kirschner and Gerhart, 1998; Tollrian and Harvell, 1999; Nijhout, 1999; Kuijper and Hoyle, 2015).

Developmental plasticity is ubiquitously observed in animals and plants and has been suggested to facilitate phenotypic diversity (West-Eberhard, 2003). However, little is known about the genetic control of developmental plasticity and its molecular mechanisms. What is the molecular nature of developmental switches controlling developmental plasticity? How do the regulation and the evolution of these mechanisms work? More mechanistic studies are necessary to fully understand the mechanisms of plastic trait determination and to illuminate the role of phenotypic plasticity in development and evolution.

1.3 *Pristionchus pacificus* from a satellite model organism to a model organism

As indicated above, evo-devo is a fusion of two disciplines. Consequently, in the early stages of evo-devo classical model organisms of developmental biology were important to obtain inside into the evolution of developmental processes. Indeed, studying model organisms was crucial in establishing evo–devo as a new discipline. However, it has been claimed that this small number of model organisms can be misleading regarding the general pattern of the role of development in evolution (Jenner and Wills, 2007). How general is the information that we obtain by studying a model organism? How conserved are patterns and processes and how conserved are individual genes and signaling pathways? Often studies in developmental biology are done in only one species and often only in one laboratory strain. To obtain a better overview of the generalizing of molecular principles of development in evolution, the phylogenetic coverage must be increased by sampling organisms with different characteristics from those of the established model organisms (Bolker, 1995; Sommer, 2005). In principles, representativeness must be determined for each molecular and mechanistic aspect a new, accounting for phylogenetic relatedness and different rates of evolutionary change in different taxa. Model organisms are more attractive for researchers if experimental techniques, genomic and genetic resources are available for a given model. However, model organisms should be good representatives of their taxa (Burian, 1993; Jenner and Wills, 2007).

C. elegans was the first nematode species established as a model organism (Brenner, 1974). This small (approximately 1 mm), transparent worm has a generation time of 3.5 days. In the laboratory it can grow on agar plates using *Escherichia coli* as food. *C. elegans* was an easy object to study the cell lineages from the zygote to the differentiated cells in the adult stage (*e.g.* Sulston and Horvitz 1977; Sulston and Horvitz, 1981). *C. elegans* was the first eukaryotic organism in which the green fluorescent protein (GFP) was expressed and proposed that it can be used to monitor gene expression and protein localization

in model organisms (Chalfie *et al*., 1994). *C. elegans* was also the first metazoan organism that had its genome fully sequenced (*C. elegans* sequencing Consortium) and the only organism whose connectome (neuronal connections) is completely mapped (White *et al*., 1986; Jabr, 2012). Several developmental processes have been studied in *C. elegans* such as vulval development, the formation of the egg-laying organ. The combination of cell lineage analysis and experimental approaches with genetic and molecular studies indicated how signaling pathways control postembryonic development. The analysis of *C. elegans* vulva development is therefore a paradigm in developmental biology (Sternberg, 2005).

Pristionchus pacificus was established as a satellite model organism in evo-devo, particularly to study vulva development in comparison to *C. elegans* (Sommer and Sternberg, 1996; Hong and Sommer, 2006). This comparative analysis surprisingly indicated that many mechanistic principles of vulva development have changed between *C. elegans* and *P. pacificus* (Sommer, 2006). The *P. pacificus* vulva is morphologically, cellularly and functionally identical to the *C. elegans* vulva (Sommer, 2006). However, the molecular mechanisms underlying developmental programs are different, providing an example for developmental systems drift (Wang and Sommer, 2011). Most importantly, *C. elegans* vulva development is induced by EGF/RAS signaling, whereas in *P. pacificus* an unconventional Wnt signaling pathway has taken the same function (Sternberg, 2005; Wang and Sommer, 2011).

More generally, *P. pacificus* has a number of advantages similar to *C. elegans* that make it a popular laboratory model, such as the small body size (up to 1 mm in length), the fact that it is easily maintained under laboratory conditions again using *E. coli* as a food source, the short life cycle, the possibility of cryopreservation, and its transparency. Also, the reproductive mode is similar to *C. elegans*. A culture consists mostly of self-fertilizing hermaphrodites (XX), and rare males (XO) that are the result of X chromosomal non-disjunction (Sommer *et al*., 1996). *P. pacificus* was first isolated from soil, and was hence described as a free-living worm (Sommer *et al*., 1996). Later, it was discovered that *P. pacificus*

nematodes live in necromenic association with scarab beetles in the wild (Herman *et al.* 2006; Hong and Sommer 2006; Herman *et al.*, 2007).

P. pacificus has several technical features that facilitate integrating development and evolution with mechanistic studies. The genome is sequenced (Dieterich *et al.*, 2008), and a genetic linkage map is available (Srinivasan *et al.*, 2002). Also, forward and reverse genetic (Zheng *et al.*, 2005, Tian *et al.*, 2008, Witte *et al.*, 2015), in-situ hybridization (Tian *et al.*, 2008) and transgenic (Schlager *et al.*, 2009) tools can be applied in an evolutionary context given the availability of more than 600 *P. pacificus* wild isolates that are cultured in the laboratory. Also, the existence of a robust phylogenetic framework of about 30 *Pristionchus* species is a useful tool for evolutionary studies (Kanzaki *et al.*, 2012a; 2013a; 2013b; Ragsdale *et al.*, 2013b; 2014; Sommer and McGaughran, 2013). One particularly important species is *P. exspectatus*, the sister species of *P. pacificus*. Both species can successfully been crossed and they produce viable but sterile hybrids (Kanzaki *et al.*, 2012a).

1.4 *P. pacificus* mouth dimorphism as an example of phenotypic plasticity

Pristionchus and other species in the family Diplogastridae, show a case of developmental plasticity in their stomatal structures (Hirschman, 1951; von Lieven and Sudhaus, 2000). *P. pacificus*, like most *Pristionchus* species, live in necromenic association with scarab beetles in their natural habitats. On the living beetle *P. pacificus* is known to occur in the arrested dauer larva (alternative resistance juvenile) stage and continues its development only after the death of the beetle in the presence of microbial food (Herman et al., 2006; Herman et al., 2007; Weller *et al.*, 2010). This 'necromenic' life style of *P. pacificus* is facilitated by the fact that these nematodes are omnivorous feeders of bacteria, fungi and other nematodes (Bento *et al.*, 2010). The feeding diversity relies on dimorphic feeding structures, which is an example of phenotypic plasticity (Bento *et al.*, 2010).

P. pacificus shows two distinct adult mouth-forms, a stenostomatous (St, 'narrow-mouthed') and a eurystomatous (Eu, 'wide-mouth') form (Fig.1). Eu and St worms differ not only by the width of their mouth, but also in the shape, the number of associated denticles and the size and form of the buccal cavity. Both, St and Eu animals have a dorsal tooth (Fig.1), which differs in shape between forms. In Eu animals this tooth is claw-like (Fig.1b), whereas in St animals it is flint-like (Fig.1a). Additionally, Eu animals have also an opposing subventral tooth (Fig.1b), which is absent in St

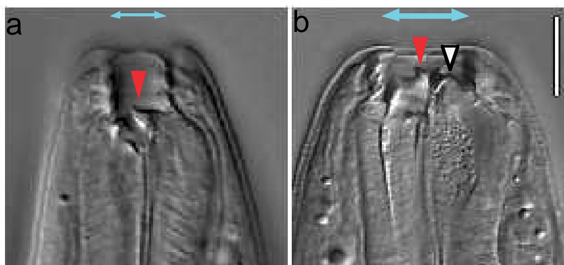


Fig.1. Mouth-dimorphism in *P. pacificus*. (a) Stenostomatous (St) animals have narrow buccal cavity (blue double arrow) and flint-like dorsal tooth (red arrow) **(b)** Eurystomatous (Eu) animals have wide buccal cavity (blue double arrow) and claw-like dorsal tooth (red arrow), additionally, they have subventral tooth (white arrow). Scale bar: 20 µm.

animals.

The mouth dimorphism of *P. pacificus* is executed in response to an irreversible developmental decision during larval development (Bento *et al.*, 2010). The exact ratio of two mouth-forms is influenced by environmental factors including starvation and culture density (Bento *et al.*, 2010). Also, it has been shown that there is some level of natural variation among different wild isolates of *P. pacificus* (Ragsdale *et al.*, 2013a), some of which is highly plastic even under stabile laboratory conditions.

P. pacificus mouth-form plasticity was first coupled with known developmental pathways. Specifically, it was shown that pheromone signaling and a conserved endocrine signaling system, consisting of the nuclear-hormone receptor DAF12 and its sterol ligand Δ7-dafachronic acid (DA), a steroid hormone that inhibits the formation of the dauer stage, influence the mouth-form ratio (Bento *et al.*, 2010; Bose *et al.*, 2012). However, mechanisms of mouth dimorphism and dauer development do not fully overlap, as the dauer-promoting transcription factor DAF-16/FOXO has no effect on the mouth phenotype (Ogawa *et al.*, 2011).

It is important to note that all environmental conditions, such as starvation and population density or pheromone and hormone treatment, can only shift the ratio of the two forms. In contrast, a complete switch was never observed in any of these studies (Bento *et al.*, 2010; Bose *et al.*, 2012). However, using forward genetics, it was possible to isolate mutant lines that were exclusively St (Ragsdale *et al.*, 2013a). Ragsdale and colleagues called such phenotypes *eu*rytomatous-form *d*efective (*Eud*). They discovered that some of the mutant lines with the *Eud* phenotype have SNPs at the same locus on the X chromosome and they named this locus *eud-1*. Interestingly, *eud-1* seems to be a specific regulator of adult mouth-form plasticity, as all mutant lines did not show any additional phenotypes. *eud-1* encodes a sulfatase and controls the developmental decision between Eu and St mouth-forms. The fact that *eud-1* mutant lines are completely St and over-expression of this gene results in completely Eu transgenic lines, indicates that *eud-1* is part of a genetic network

that constitutes the developmental switch of the *P. pacificus* mouth-form decision. Epistasis experiments showed that *eud-1* acts downstream of, or in parallel to, pheromone and hormone signaling and is expressed in head neurons. Interestingly, *eud-1* acts as switch gene not only in *P. pacifivus* but also in its sister species *Pristionchus expectatus*. However, there are regulatory differences between two species with both their X chromosome and autosomal background, suggesting rapid evolution of the regulatory mechanism for an otherwise conserved developmental switch (Ragsdale *et al*., 2013a).

1.5 Nematodes as a model for studying epigenetic mechanisms and developmental plasticity

Epigenetic processes have emerged as a diverse and important collection of mechanisms that mediate the interaction between the environment and the genome at multiple levels, enabling the expression of developmentally plastic phenotypes (Gilbert and Epel, 2009; Beldade *et al.*, 2011; Valena and Moczek, 2012). Studies of insect, mammalian and plant model organisms have provided powerful insights into the nature and consequences of epigenetic mechanisms.

Technical features of nematodes make them attractive models for researchers that study molecular biology and developmental genetics. However, until recently it was considered that DNA methylation is absent in *C. elegans* because of the lack of detectable 5-methylcytosine (Simpson *et al.*, 1986) and because the isolation and culturing of cells was limited, making nematodes less attractive for studying epimutations and mechanisms of epigenetic processes. Recently, epigenetic inheritance and its mechanisms were under the focus of researchers that are interested in sex determination and dosage compesation of roundworms, and transgenerational inheritance mediated by small RNAs (e.g. Meyer, 2000; Johnson and Spence, 2011; Greer *et al.*, 2011; Rechavi et *al.*, 2011; Strome *et al.*, 2014; Tabuchi *et al.*, 2014). Most strikingly, the recent discovery of DNA methylation on N6-Adenine in *C. elegans*, the description of its role in epigenetic inheritance (Greer *et al.*, 2015) and the development of a method for large-scale isolation and primary culture of *C. elegans* larval-stage cells (Zhang *et al.*, 2011) has opened new avenues for studying epigenetic mechanisms in roundworms. These methodological advantages will allow researchers to study transgenerational effects and epigenetic modifications in a tissue specific manner as well as at the organism level. Such studies might detect a link to plasticity and its underlying developmental mechanisms.

1.6 Aim of the thesis

My work discussed in this thesis contributes to a systematic characterization of the mouth-form plasticity of *P. pacificus*. Specifically, I show that plasticity is sex-linked in *P. pacificus* and that there is a correlation between the mothers' mouth-form and that of their male progeny in an inbred genetic background. In contrast, I show that such a correlation does not exist in hermaphrodite progeny. Also, there is an influence of conspecifics on developmental decision. Furthermore, I show that the two forms show conditional fitness advantages in different environments. Particularly, St animals develop faster when unlimited bacterial food is provided, whereas Eu animals have an advantage in predatory beahaviour, which is beneficial when bacterial food is missing.

The main objective of my thesis focuses on the analysis of the genetic basis and the molecular mechanisms controlling the developmental switch that regulates phenotypic plasticity. I show that mouth-form plasticity is under strong epigenetic control including chromatin remodeling and lncRNA-mediated gene expression. One important aspect is the analysis of the genetic basis responsible for the correlation between maternal mouth-form and the mouth-form of male progeny as well as the sex-linked phenomenon of plasticity in general.

2 Results and Discussion

2.1 Feeding plasticity in the nematode *Pristionchus pacificus* is influenced by sex and social context and is linked to developmental speed

Vahan Serobyan, Erik J. Ragsdale, Manuela R. Müller and Ralf J. Sommer

Evolution & Development (2013) 15:3, 161–170

2.1.1 Synopsis

Theoretical and empirical evidence suggests that developmental plasticity in response to changing environmental conditions facilitates the evolution of novel traits at the morphological level. In the nematode *Pristionchus pacificus*, a developmental plasticity-affecting structure important for feeding can be studied under laboratory conditions. During development, worms either form a stenostomatous (St, 'narrow-mouthed') or a eurystomatous (Eu, 'wide-mouthed') mouth-form that differ in several characters. Intermediates are usually rare. Both forms are consistently present in populations, and recent molecular studies have begun to provide insight into the molecular processes regulating this mouth-form decision. In this study we provide systematic characterization of feeding-structure plasticity in the *P. pacificus* reference strain, which provides a baseline of understanding mouth-form plasticity as an essential reference point for further studies.

By quantifying the mouth-form phenotype of spontaneous males (that are result of X chromosome non-disjunction) and their cohort hermaphrodites, we found that mouth-form plasticity has a strong sexual dimorphism in *P. pacificus* and that in an inbred genetic background the maternal phenotype is correlated with that of male progeny. In particular, the St form is rare in our reference strain hermaphrodites. In contrast, the St form is the predominant form in males.

Crosses between spontaneous males and their cohort hermaphrodites showed that the phenotype of their sons, but not that of their daughters, depends on the phenotype of the mother. Such a correlation between mothers and their male progeny cannot be explained purely by genetic inheritance. Therefore, we explain such a maternal influence by cross-generational epigenetic changes, which can be due to possible hormonal cues that are transferred from the mother to male progeny *in utero*. Moreover, isolating juveniles from culture in different developmental stages resulted in lower Eu frequencies, which decreased gradually with earlier isolation. Also, St hermaphrodites developed faster Eu hermaphrodites when abundant bacterial food was provided, suggesting a possible fitness trade-off between developmental time and diet.

2.1.2 Own Contribution

I performed all the experiments in collaboration of Erik J. Ragsdale and Manuela R. Müller. I contributed to the experimental design and analyses and to the writing of the manuscript. My contribution to this publication is 90%.

2.2 Adaptive value of a predatory mouth-form in a dimorphic nematode

Vahan Serobyan, Erik J. Ragsdale and Ralf J. Sommer

Proceedings of the Royal Society of London B (2014) Volume: 281 Issue: 1791

2.2.1 Synopsis

Phenotypic plasticity, the ability of an organism to change its phenotype in response to changing environmental conditions is increasingly considered to be an important source of selectable variability in evolutionary diversification. The nematode *P. pacificus*, a laboratory model for comparative mechanistic biology, shows phenotypic plasticity in its feeding structures and has therefore emerged as a model for investigating the interface between trophic interactions and developmental mechanisms in evolution. The plasticity of *P. pacificus* is a resource polyphenism that responds to and takes advantage of changing food resources. In particular, *P. pacificus* is able to feed as a predator, a trophic novelty with respect to related nematodes that lack the feeding plasticity. The feeding structures of *P. pacificus* constitute a structural innovation and occur as a dimorphism in the adult stage, namely as either a stenostomatous ("narrow mouthed," St) or a eurystomatous ("wide mouthed," Eu) form, both of which differ in the number and shape of teeth. The developmental decision between these two forms controls the level of expression of a single gene.

Under conditions of starvation and crowding (pheromone signaling), populations show an increase in the frequency of the Eu form, which was previously only assumed to be associated with omnivorous feeding. Additionally, in a previous study it was discovered that St animals develop faster compared to Eu animals in an environment where unlimited bacterial food was provided. In this study we investigate a possible fitness trade-off of Eu animals. We show that on a prey diet *P. pacificus* Eu hermaphrodites, in contrast to St hermaphrodites,

indeed show greater fitness including longevity, offspring survival and fecundity when followed by bacterial feeding. Nevertheless, both forms show a similar number of progeny when they feed on bacteria, an environmental condition that is beneficial for St form with respect to developmental timing. Together with our previous findings this study provides a functional context of the dimorphism: both Eu and St forms have benefits in different environments.

2.2.2 Own Contribution

I contributed to the experimental design, performed the research, and contributed to analyses of data and to the writing of the manuscript. My contribution to this publication is 90%.

2.3 Chromatin remodeling and antisense-mediated up-regulation of the developmental switch gene *eud-1* control a predatory feeding decision

Vahan Serobyan, Hua Xiao, Christian Rödelsperger, Suryesh Namdeo, Waltraud Röseler, Hanh Witte and Ralf J. Sommer

Nature Communications (submitted 2015)

2.3.1 Synopsis

Developmental plasticity is the ability of a genotype to execute a phenotype to adapt rapidly to a changing environment. Polyphenisms as a special case of developmental plasticity, in particular, comprise alternative phenotypes that can be functionally independent and which result from developmental switches. The nematode *Pristionchus pacificus*, a laboratory model for mechanistic genetic studies, shows a polyphenism in its feeding structures and hence has emerged as a model for mechanisms regulating plasticity. This species shows a developmentally irreversible dimorphism in its mouthparts, which take either a stenostomatous ("narrow mouthed," St) or eurystomatous ("wide mouthed," Eu) form. The dimorphism is also sex-linked, such that hermaphrodites (morphological females) are highly Eu and males highly St. The developmental decision between these two forms controls the level of expression of a single gene on the X chromosome: *eud-1*, which encodes a sulfatase. Although *eud-1* is known to be part of a developmental switch and functions in a dosage-dependent manner, the molecular mechanisms controling *eud-1* expression are still unknown. In this study, we show that *eud-1* is under strong epigenetic control.

Mutations in two genes, *Ppa-lsy-12* (encoding a histone acetyltransferase) and *mbd-2* (encoding a methyl-binding domain) show a *Eu*-form-*d*efective (*Eud*) phenotype, and *eud-1* expression is down-regulated in both mutant lines. Mutations in both genes show histone modification defects, involving H3K4me3,

H3K4me2, H3K9ac, and H3K27ac. Interestingly, RNA-seq experiments show down-regulation of an antisense-*eud-1* long non-coding RNA in *lsy-12* mutants. Over-expression of this RNA in a wild-type background shows *h*igh *i*ncidence of *m*ales (*Him*), including an increased number of Eu animals. In contrast, over-expression of the same construct in a *eud-1* background results in only a *Him* phenotype with no rescue of the Eu form. Taken together, our analysis of mutants for histone modification suggests *as-eud-1* to be a positive regulator of *eud-1*.

2.3.2 Own Contribution

Hua Xiao, a former graduate student, performed physical cloning of *vul-2*. Christian Rödelsperger carried out computational analyses. Hanh Witte performed some microinjection experiments, and Waltraud Röseler was involved in *Ppa-lsy-12* RACE experiments. Suryesh Namdeo was involved in RNA FISH experiments. I performed all other experiments. My contribution to this study corresponds to 70%.

2.4 Mapping, cloning and characterization of genes regulating *P. pacificus* mouth-form dimorphism in males

2.4.1 Introduction

The mouth dimorphism in *P. pacificus* is sex linked. Hermaphrodites (XX) have a 70-90% Eu frequency, whereas spontaneous males (XO) are approximately 90% St (Ragsdale *et al.*, 2013a; Serobyan *et al.*, 2013). Surprisingly, a maternal effect results in male offspring of crosses between Eu mothers and St fathers to be 20-30% Eu. In contrast, male offspring of St parents are nearly all St (Serobyan *et al.*, 2013). Such a maternal influence cannot completely be explained by genetic inheritance. Therefore, generating mutant lines that are defective in the maternal influence opens the possibility to study maternal effect in the context of developmental plasticity at the individual gene level.

2.4.2 Genetic screen for maternal-infuence defective phenotype

To study the maternal and transgenerational effect and potentially associated epigenetic control of *P. pacificus* mouth-form plasticity, I screened for mutants that abandoned the maternal effect in males. Specifically, the F2 progeny of mutagenized hermaphrodites were crossed to St males in search for highly-Eu male progeny (Fig.2a). From a mutagenesis screen of 1500 haploid gametes, I identified two mutant lines that resulted in highly-Eu male progeny and called these mutants *mid-1* and *mid-2* for *m*aternal-*i*nfluence *d*efective (Fig.2b). Both mutant lines have a complex phenotype including strong *unc*oordinated movement (*Unc*). I could not separate *Mid* and *Unc* by backcrossing experiments, neither in *mid-1* nor in *mid-2*. This finding indicates that either there is one mutation that is causing both aspects of the phenotype or there are two different mutations that are located closely to each other in the genome, the latter of which is very unlikely.

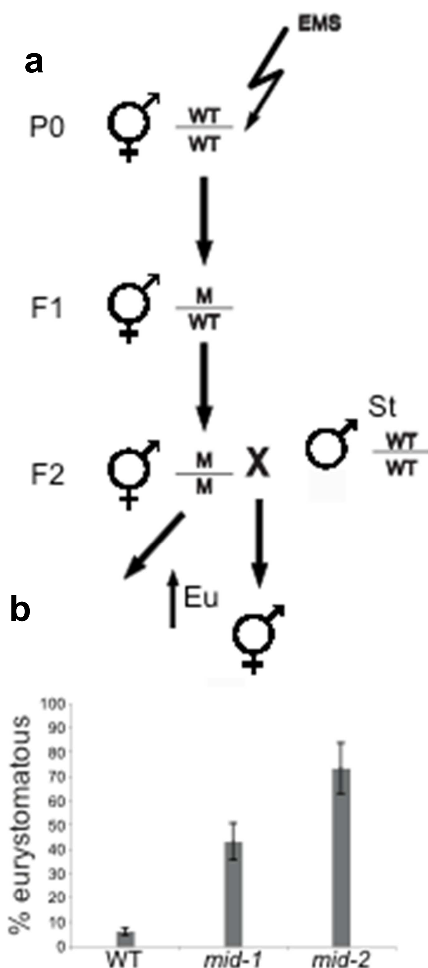


Fig.2. Genetic screen for *m*aternal *i*nfluence *d*efective mutants and the mouth-form ratio of male progeny in wild type and *Mid* mutants. **(a)** Wild type (RS2333) worms were mutagenized with ethyl methansulfonate (EMS) and allowed to self-fertilize for two generations. Adult St animals (F2) were transferred to new plates with wild type males. The cross-male-progeny was screened for the phenotype < 20% Eu. **(b)** Two mutant line *mid-1* (*tu513*) and *mid-2* (*tu514*) show maternal influence defective phenotype. Data are presented as the total Eu frequency n>30 for all strains.

Using simple-sequence length and conformation polymorphism markers, I mapped both of these recessive mutants. The *mid-1* mutation was mapped to chromosome III (Fig.3a) and the *mid-2* mutation to the X chromosome (Fig.4a). For the details of fine mapping see Material and Methods.

2.4.3 *mid-1* encodes a SR proteine kinase

Mapping results indicted that *mid-1* is located in a 750kb interval of contig2 on chromosome III (HYBRID1). I generated and sequenced whole-genome libraries and searched for SNPs in this 750kb region. There were only two SNPs, both of which where confirmed by Sanger sequencing. Only one of the SNPs was

in a predicted exon. Therefore, I performed RACE experiments to obtain the full structure of the associated gene (Contig2-snap.208) and confirmed the predicted exon-intron structure. I identified two different isoforms of Contig2-snap.208 (Fig.3b). Indeed, further sequencing revealed that one of the isoforms, contains the SNP resulting in a nonsynonymous amino acid change. However, the exon with the mutation is only present in one isoform. The cytosine to guanine mutation is causing an amino acid substitution from threonine to methionine.

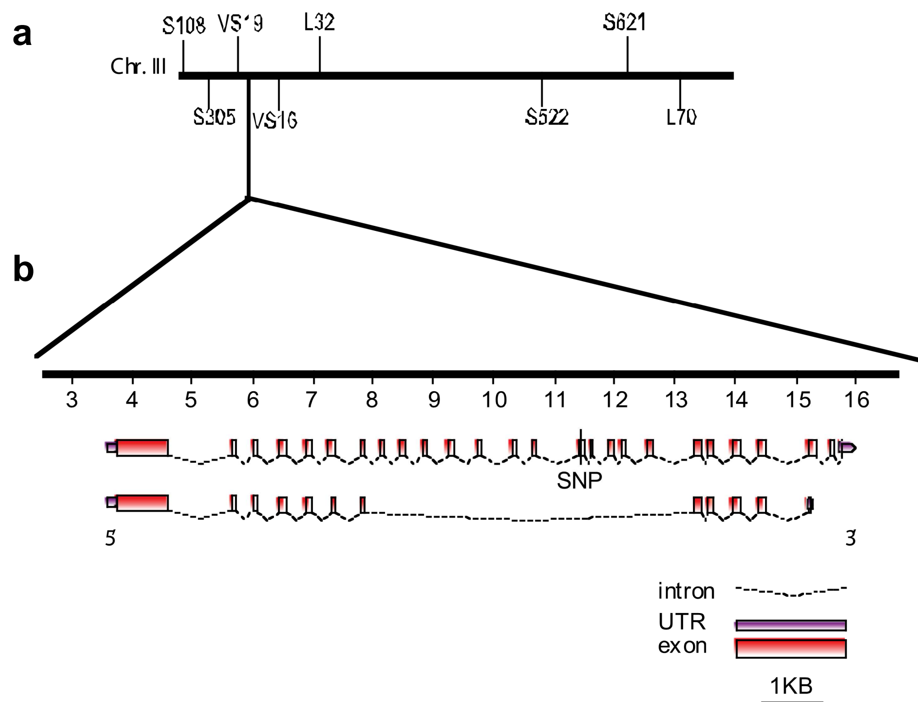


Fig.3. Mapping and gene structure *Ppa-spk-1*. (a) Genetic map of a region containing *mid-1* locus on the chromosome **III**. **(b)** *Ppa-spk-1* gene structure, isoforms and the SNP

Contig2-snap.208 encodes a serine/arginine-rich protein kinase that by gene ontology phosphorylates SR proteins, an essential component of the pre-mRNA splicing machinery (Kuroyanagi *et al.*, 2000). This *P. pacificus* gene is orthologous to the *C. elegans* gene *spk-1*.

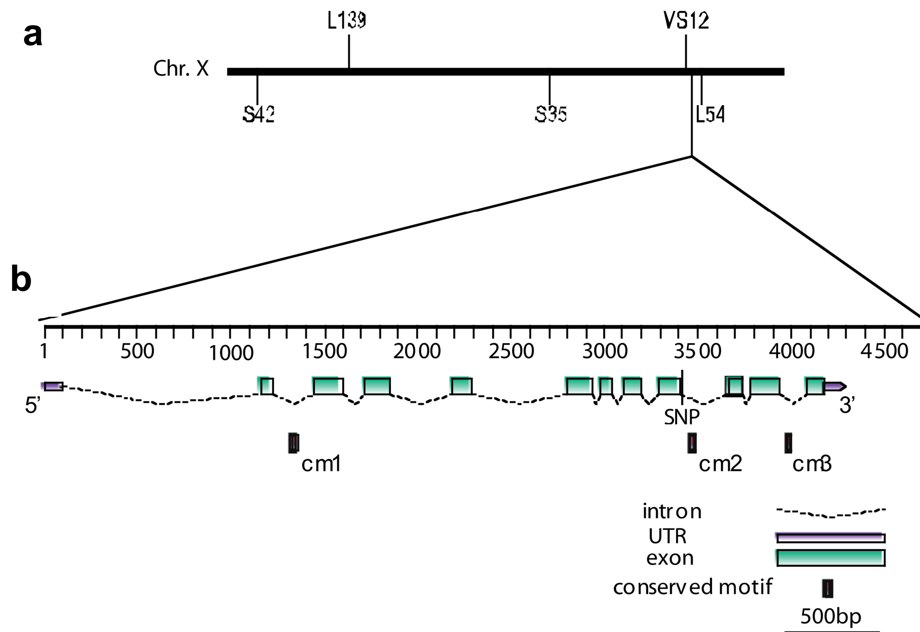


Fig.4. Mapping and gene structure of *mid-2* locus. (a) Genetic map of a region containing *mid-1* locus on the chromosome III. **(b)** The structure of a protein coding gene and the conserved motifs indicating the presence of antisense transcript. The SNP is in the intron of the protein-coding transcript.

To attempt phenotypic rescue I generated a construct of *Ppa-spk-1* with a 3.5kb promoter region and obtained two independently transformed lines carrying the *Ppa-spk-1* construct with a *egl-20::rfp* (red fluorescent protein) reporter. Both transgenic lines recued the *Unc* and the mouth-form phenotype; specifically, the mouth-form ratio switched to 100% St for male progeny after crosses (Fig.5a). In addition, all hermaphrodites were 100% St (Fig.5a). This result indicates that *Ppa-spk-1* causes the original *mid-1* phenotype and therefore the gene was renamed accordingly.

To test whether the effect on hermaphrodite is a result of overexpression *of Ppa-spk-1* by the extrachromosomal array, I overexpressed the gene also in a wild type background. Indeed, *Ppa-spk-1* overexpression (OE) resulted again in 100% St hermaphroditic animals (Fig.5a).

As the maternal effect is observed in males I predicted that *Ppa-spk-1* might act through the developmental switch gene *eud-1*, which is X-linked and known to be downregulated in males (Ragsdale *at al.*, 2013a). Therefore, I next

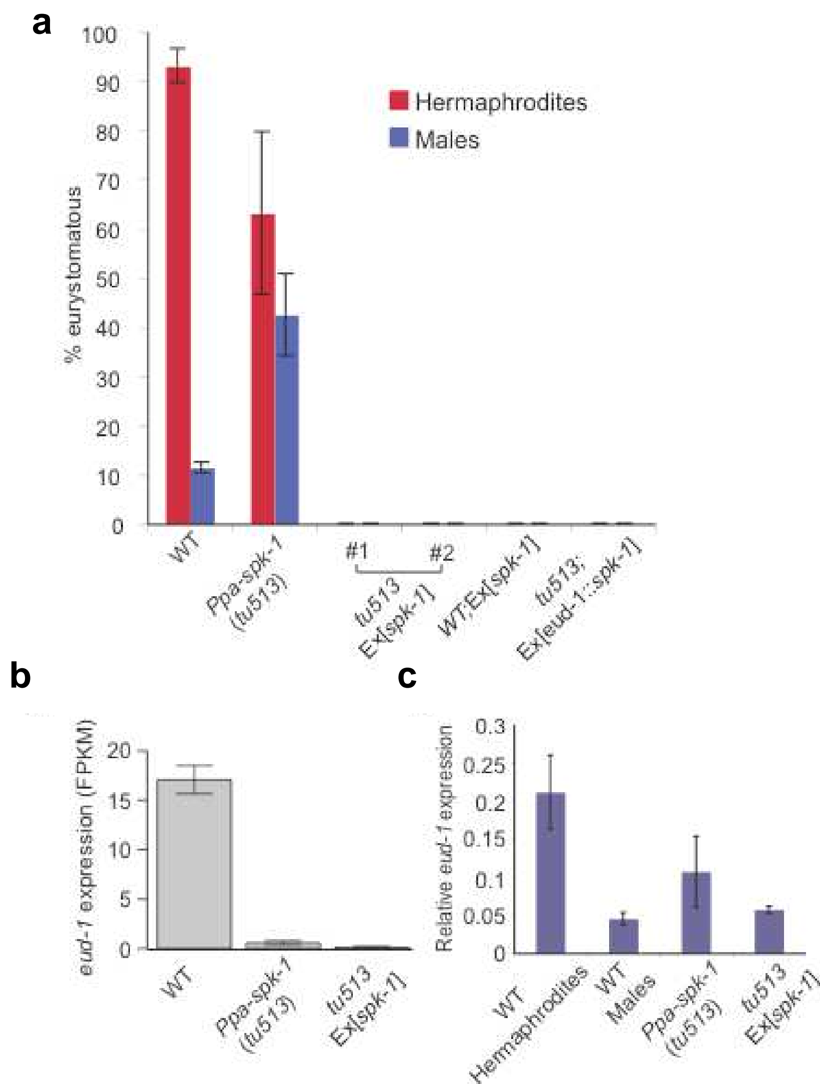


Fig.5. *Ppa-spk-1* expression levels effect on *P. pacificus* mouth-form plasticity in males and hermaphrodites. (a) The mouth-form ratio of St parent progeny (male and hermaphrodite). Independently from background *Ppa-spk-1* overexpression results *Eud* phenotype. (b) RNA-seq shows down regulation of *eud-1* in both *Ppa-spk-1* and rescued line (mixed cultures). **(C)** *eud-1* expression levels in wild type hermaphrodites, males, *Ppa-spk-1* (*tu513*) mutant and *Ppa-spk-1* overexpressed line.

analyzed *eud-1* expression levels in *Ppa-spk-1*-OE lines and *Ppa-spk-1* mutant lines. Both, qRT-PCR and RNA-seq experiments showed downregulation of *eud-1* in *Ppa-spk-1*-OE lines (Fig.5b,c). Next, I created a construct, which contains a 7.5kb promoter region of *eud-1* fused to the long transcript of *spk-1* and the 3' UTR. A transgenic line of this construct in a *mid-1* background was still *Unc*, but had a *Eud* phenotype (Fig5a). This finding suggests that if *spk-1* is expressed in

the same cells that normally express *eud-1*, it induces the Eu moth-form. We speculate that *Ppa-spk-1* acts upsteam of *eud-1* and regulates splicing or alternative splicing of an unknown factor that directly or indirectly act through *eud-1* itself. Interestingly, when I performed RT-PCRs amplifying full cDNA from *Ppa-spk-1* mutant and wild type animals, I found *eud-1* and *lsy-12* to show defects in alternative splicing in *Ppa-spk-1* (data not shown). This finding has to be investigated further to reach full conclusions.

2.4.4 *mid-2* might encode a lncRNA

Mapping results indicated *mid-2* to be located in a 370kb interval of contig4 on the X chromosom (HYBRID1). Genome sequencing of the mutant line showed only two SNPs in this region, and both SNPs have been confirmed by Sanger sequencing. Interestingly, both SNPs were in the introns of the predicted genes (Contig4-snap.351 and Contig4-snap.317). I performed rescue experiment for both genes, and found only partial rescue with a construct containing Contig4-snap.317, resulting in strange looking F1 sterile males that had no *Unc* phenotype. Contig4-snap.317 encodes a predicted protein with unknown structure and function. Further analysis of the locus indicated that there is an antisense transcript expressed from the locus that contains the SNP (Fig.4b). Interestingly, this antisense transcript has three conserved motifs and two of them match sequence reads of small RNA libraries. One of these conserved motifs is identical to the second exon of as-*eud-1* that is 26 bp long. Together, all these results suggest that *mid-2* might act via the switch gene *eud-1* and that *mid-2* regulates *eud-1* expression as an epigenetic factor. The project is currently continuing by Suryesh Namdeo, a new graduate student in the laboratory.

2.4.5 Material and methods

Culture Conditions

P. pacificus RS2333 was kept on 6-cm plates with nematode growth medium (NGM) agar and was fed with a lawn of *E. coli* OP50 grown in 300-400 µl L-Broth. For crosses, 6-cm NGM agar plates were seeded with a lawn OP50. Cultures were maintained at 20°C to avoid unknown factors that can effect the mouth-form ratio.

Phenotypic scoring

Characters used to distinguish between Eu and St individuals, respectively, were (1) the presence *vs*. absence of a subventral tooth, (2) a claw-like *vs*. flint-like or triangular dorsal tooth, and (3) a wide *vs*. narrow stoma (mouth). Intermediates between the two forms were rare (<0.1%) and were not included in counts. Mostly, phenotypes were scored using Zeiss Discovery V.12 stereomicroscope and new lines were additionally screened by differential interference contrast (DIC) microscope on a Zeiss Axioskop.

Genetic screen for ***m**aternal-**i**nfluence-**d**efective* phenotype

RS2333 mixed stage worms were mutagenized with ethyl methansulfonate (EMS) as described by Pires da Silva (2006) and allowed to self-fertilized for two generations. F2 animals were transferred in the J4 stage to a new plate and were incubated for two days to lay eggs. F2 animals that showed a St mouth-form were transferred to new plates for mating with wild type males. Original plates with ‘clones’ of these mothers were used as voucher. After giving cross progeny, all males on the plates were screened for mouth-form phenotypes (Fig.2a).

Mapping

Mutant hermaphrodites in the California (RS2333) background were crossed with males of the Washington strain (PS1843) (Srinivasan, 2002). F2

animals were transferred in the J4 stage to new plates to lay eggs as a voucher and were crossed with California males for phenotype screening. Genomic DNA was extracted from voucher-plates. To assign linkage of the mutation to a certain chromosome, SSLP and some SSCP markers were used. 30 lines for *mid-1* and 44 lines for *mid-2* were tested using Washington double-backcrossed animals. SSLP detection was performed on 4% agarose gel. For SSCP detection PCR products were denaturated at 95°C for 5 minutes and loaded onto a GeneGel Excel prepoured 6% acrylamide gel (PharmaciaBiotech). To narrow down the physical region of the candidate genes, new SSCP markers were designed (Fig.3a; Fig.4a; Table 1).

Table 1. List of newly designed SSCP markers

Marker	contig (HYBRID1)	Chromosome	Primer	Forward	primer	Reverse
VS9	contig4	X	vs26161	GCGGTGCAGTAAAGAGAAATG	vs26162	GTTCCGTTCTCCCCTAATTGA
VS12	contig4	X	vs26167	GTCCGGAGCCTCGCTCCCT	vs26168	TTCACCTTGTCATTGCTTTCGCTGA
VS13	contig4	X	vs26169	CTCCGGAGAGTTCCCATCTCAACG	vs26170	GCCCTCCCTCGCCCTCGTAA
VS15	contig4	X	vs26173	TCGACAACTTCGGTGTGCGAGC	vs26174	AGCCACCCCTCGAACCGTCC
VS16	contig2	III	vs26294	AAGCCACAGTTGACCGAAAT	vs26295	CGATTTTCTCCCAGCACTTC
VS17	contig2	III	vs26296	CGATTCTCCTTGGCGTAGAG	vs26297	TGTCTCGTTGATGGGTGAAA
VS18	contig2	III	vs26298	GATTACCAATTGTTCGCTCCA	vs26299	CAGCGAATGTGGTCGTTAAAT
VS19	contig2	III	vs26300	TCGAGCGAGGCCAATTCCGC	vs26301	CCGCACTGGAGGCAGTGAACA
VS21	contig2	III	vs26304	CGCACAGCTATCTCAAGGATTT	vs26305	ATATATGTAGCCAGCCCACTGC

Full-genome sequencing

DNA was extracted and purified using the MasterPure DNA purification kit (Epicentre). To find SNPs in the candidate genes in mapped region full-genome sequencing was performed (illumina). All libraries were diluted to a concentration of 10 nM in 0,1% EB-Tween and pooled as 4-plex. The resulting libraries were sequenced as 100bp paired ends on an Illumina Genome Analyzer II to coverage of 9X.

Rescue experiments

Extrachromosomal arrays were generated as described by Schlager *et al.* (2009). Animals were injected mixture with genomic construct (2-5ng/µl), the marker *Ppa-egl-20*::*TurboRFP* (10ng/µl) and genomic carrier DNA (60ng/ µl) from

the recipient mutant line. DNA extraction was done by SIGMA life Science GeneElute Mammalian Genomic DNA Miniprep Kits.

RNA extraction cDNA synthesis and qRT-PCR experiments

Total RNA from synchronized cultures was isolated using TRIzol (ambion by life technologies). For reverse transcription Superscript II reverse transcriptase (Invitrogen) was used following the manufacturer's instructions. We used 1μg total RNA. The qRT-PCR experiments were performed on a LightCycler 480 system using SybrGreen (Roche Diagnostics). Reaction set up was performed according to Schuster and Sommer (2012). To detect *eud-1* expression levels, was used VSe13F GATGATCGAGTCACACAGATCCG forward and VSe13R ATGTAGTAGGAGAGTTGAGCAGCG reverse primer pair. *Ppa-ama-1*, *Ppa-cdc-42* and *Ppa-Y45F10D.4* were used as reference genes (for the primer list see Schuster, Sommer, 2012). PCR efficiencies were determined using external standards on plasmid mini-preparation of cloned PCR-product. Expression levels were analyzed by basic relative quantification and at least three biological replicates were performed.

RACE

5' and 3' RACE experiments were performed by SMARTer RACE cDNA Amplification Kit following the manufacturer's protocol. The full list of gene-specific primers that were designed according to the available genomic sequence for *Ppa-spk-1* and *mid-2* sense gene is provided in Table 2.

Table 2. List of gene specific primers used for RACE experiments

Gene	Primer	Oligo
P.pa-spk-1	Co2GSP2	GAGGGTGGCCCGCCCGTGCCTAGGCTA
	Co2NGSP2	CTGACATTTGGAGTACGGCGTGTATGG
	Co2GSP1	CGTTGCTGGGCAGGCTGTTCTCGGCTTC
	Co2NGSP1	CTCCACTCTCCTCATCTCCCTCCTCCT
mid-2	Co3GSP2	CTGACTCTGCTCCATCCGAATGCTACTC
	Co3GSP1	CGACTTCTTTCCGTCGATTTCAGGTGAG
	Co3NGSP2	TCCTGCTAGCAAAGCGAATTCACACG
	Co3NGSP1	CTCGATTCTCCGAGCATTCCACGAA

Ultradirectional RNA library preparation

Levels of gene expression were measured by whole-transcriptome sequencing (RNA-Seq) of *Ppa-spk-1 (tu319)* mutants, *P. pacificus* wild-types and *Ppa-spk-1*-OE. Culture populations were allowed to grow until their food was exhausted, when the cultures were processed for sequencing. Five mixed-stage plates were washed with 40 ml M9, centrifuged immediately at 1300 g for 4 min, rinsed with 40 ml 0.9% NaCl treated with 40 µl ampicillin and 40 µl chloramphenicol and shaken gently for 2 hours. Finally, worms were concentrated into a pellet by centrifugation and immediately frozen in liquid nitrogen. NEBNext Ultradirectional RNA Library Kit was used to prepare libraries. RNA-Seq libraries were sequenced as 2x 100-bp paired-end reads on an Illumina HiSeq 2000, yielding 11-45 million paired-end reads per sample. Raw reads were aligned to the reference genomes of *P. pacificus* (Hybrid1) (www.pristionchus.org), using the software Tophat v.2.0.3 (Trapnell *et al*., 2012). Expression levels were estimated and compared using the programs Cufflinks and Cuffdiff v.2.0.1 (Trapnell *et al*., 2012).

Gene structure illustration

The gene structure illustration was done by fancyGENE (Rambaldi and Ciccarelli, 2009).

References

Abouheif, E., and Wray, G. A. (2002). Evolution of the gene network underlying wing polyphenism in ants. *Science*, *297*(5579), 249-252.

Ahmad, K., and Henikoff, S. (2002). Epigenetic consequences of nucleosome dynamics. *Cell*, *111*(3), 281-284.

Allison, S. J., and Milner, J. (2014). RNA Interference by Single-and Double-stranded siRNA With a DNA Extension Containing a 3′ Nuclease-resistant Mini-hairpin Structure. *Molecular Therapy—Nucleic Acids*, *3*(1), e141.

Amir, R. E., Van den Veyver, I. B., Wan, M., Tran, C. Q., Francke, U., and Zoghbi, H. Y. (1999). Rett syndrome is caused by mutations in X-linked MECP2, encoding methyl-CpG-binding protein 2. *Nature genetics*, *23*(2), 185-188.

Anderson, N. L., Anderson, N. G., Pearson, T. W., Borchers, C. H., Paulovich, A. G., Patterson, S. D., ... and Carr, S. A. (2009). A human proteome detection and quantitation project. *Molecular and cellular proteomics*, *8*(5), 883-886.

Arthur, W. (2000). Intraspecific variation in developmental characters: the origin of evolutionary novelties. *American Zoologist*, *40*(5), 811-818.

Bachman, K. E., Park, B. H., Rhee, I., Rajagopalan, H., Herman, J. G., Baylin, S. B., Kinzler K. W. and Vogelstein, B. (2003). Histone modifications and silencing prior to DNA methylation of a tumor suppressor gene. *Cancer cell*, *3*(1), 89-95.

Bannister, A. J., and Kouzarides, T. (2011). Regulation of chromatin by histone modifications. *Cell research*, *21*(3), 381-395.

Barriocanal, M., Carnero, E., Segura, V., and Fortes, P. (2014). Long non-coding RNA BST2/BISPR is induced by IFN and regulates the expression of the antiviral factor Tetherin. *Frontiers in immunology*, *5*.

Basnet, H., Su, X. B., Tan, Y., Meisenhelder, J., Merkurjev, D., Ohgi, K. A., ... and Rosenfeld, M. G. (2014). Tyrosine phosphorylation of histone H2A by CK2 regulates transcriptional elongation. *Nature*.

Batista, P. J., and Chang, H. Y. (2012). Cytotopic localization by long noncoding RNAs. *Current opinion in cell biology*, *25*(2), 195-199.

Batista, P. J., and Chang, H. Y. (2013). Long noncoding RNAs: cellular address codes in development and disease. *Cell*, *152*(6), 1298-1307.

Baylin, S. B., Fearon, E. R., Vogelstein, B., de Bustros, A., Sharkis, S. J., Burke, P. J., ... and Nelkin, B. D. (1987). Hypermethylation of the 5'region of the

calcitonin gene is a property of human lymphoid and acute myeloid malignancies. *Blood*, *70*(2), 412-417.

Becker, C., Hagmann, J., Müller, J., Koenig, D., Stegle, O., Borgwardt, K., and Weigel, D. (2011). Spontaneous epigenetic variation in the Arabidopsis thaliana methylome. *Nature*, *480*(7376), 245-249.

Beldade, P., Mateus, A. R. A., and Keller, R. A. (2011). Evolution and molecular mechanisms of adaptive developmental plasticity. *Molecular Ecology*, *20*(7), 1347-1363.

Bell, A. C., and Felsenfeld, G. (2000). Methylation of a CTCF-dependent boundary controls imprinted expression of the Igf2 gene. *Nature*, *405*(6785), 482-485.

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

Berger, S. L. (2007). The complex language of chromatin regulation during transcription. *Nature*, *447*(7143), 407-412.

Berger, S. L., Kouzarides, T., Shiekhattar, R., and Shilatifard, A. (2009). An operational definition of epigenetics. *Genes and development*, *23*(7), 781-783.

Bernardo, J. (1996). Maternal effects in animal ecology. *American Zoologist*, *36*(2), 83-105.

Bestor, T. H., and Ingram, V. M. (1983). Two DNA methyltransferases from murine erythroleukemia cells: purification, sequence specificity, and mode of interaction with DNA. *Proceedings of the National Academy of Sciences*, *80*(18), 5559-5563.

Bird, A. P., and Wolffe, A. P. (1999). Methylation-induced repression—belts, braces, and chromatin. *Cell*, *99*(5), 451-454.

Bird, A., Tate, P., Nan, X., Campoy, J., Meehan, R., Cross, S., ... and Macleod, D. (1995). Studies of DNA methylation in animals. *Journal of Cell Science*, *1995*(Supplement 19), 37-39.

Bjornsson, H. T., Fallin, M. D., and Feinberg, A. P. (2004). An integrated epigenetic and genetic approach to common human disease. *TRENDS in Genetics*, *20*(8), 350-358.

Black, D. L. (2003). Mechanisms of alternative pre-messenger RNA splicing. *Annual review of biochemistry*, *72*(1), 291-336.

Bolker, J. A. (1995). Model systems in developmental biology. *BioEssays*, *17*(5), 451-455.

Bonduriansky, R., and Day, T. (2009). Nongenetic inheritance and its evolutionary implications. *Annual Review of Ecology, Evolution, and Systematics*, *40*, 103-125.

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

Bourc'his, D., and Bestor, T. H. (2004). Meiotic catastrophe and retrotransposon reactivation in male germ cells lacking Dnmt3L. *Nature*, *431*(7004), 96-99.

Brakefield, P. M., and Reitsma, N. (1991). Phenotypic plasticity, seasonal climate and the population biology of Bicyclus butterflies (Satyridae) in Malawi. *Ecological Entomology*, *16*(3), 291-303.

Brantl, S. (2007). Regulatory mechanisms employed by cis-encoded antisense RNAs. *Current opinion in microbiology*, *10*(2), 102-109.

Brenner, S. (1974). The genetics of Caenorhabditis elegans. *Genetics*, *77*(1), 71-94.

Brookes, E., Laurent, B., Õunap, K., Carroll, R., Moeschler, J. B., Field, M., ... and Shi, Y. (2015). Mutations in the intellectual disability gene KDM5C reduce protein stability and demethylase activity. *Human molecular genetics*, ddv046.

Burian, R. M. (1993). How the choice of experimental organism matters: Epistemological reflections on an aspect of biological practice. *Journal of the History of Biology*, *26*(2), 351-367

Buschhausen, G., Wittig, B., Graessmann, M., and Graessmann, A. (1987). Chromatin structure is required to block transcription of the methylated herpes simplex virus thymidine kinase gene. *Proceedings of the National Academy of Sciences*, *84*(5), 1177-1181.

Bustin, M., Catez, F., and Lim, J. H. (2005). The dynamics of histone H1 function in chromatin. *Molecular cell*, *17*(5), 617-620.

Carrieri, C., Cimatti, L., Biagioli, M., Beugnet, A., Zucchelli, S., Fedele, S., ... and Gustincich, S. (2012). Long non-coding antisense RNA controls Uchl1 translation through an embedded SINEB2 repeat. *Nature*, *491*(7424), 454-457.

Castle, J. C., Zhang, C., Shah, J. K., Kulkarni, A. V., Kalsotra, A., Cooper, T. A., and Johnson, J. M. (2008). Expression of 24,426 human alternative splicing events and predicted cis regulation in 48 tissues and cell lines. *Nature genetics*, *40*(12), 1416-1425.

Cattanach, B. M., and Kirk, M. (2004). Differential activity of maternally and paternally derived chromosome regions in mice. *Landmarks in Medical Genetics: Classic Papers with Commentaries*, *51*, 245.

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. W., and Prasher, D. C. (1994). Green fluorescent protein as a marker for gene expression. *Science*, *263*(5148), 802-805.

Chodavarapu, R. K., Feng, S., Bernatavichute, Y. V., Chen, P. Y., Stroud, H., Yu, Y., ... and Pellegrini, M. (2010). Relationship between nucleosome positioning and DNA methylation. *Nature*, *466*(7304), 388-392.

Cole, A. J., Clifton-Bligh, R., and Marsh, D. J. (2015). Histone H2B monoubiquitination: roles to play in human malignancy. *Endocrine-related cancer*, *22*(1), T19-T33.

Cooper, D. N., and Youssoufian, H. (1988). The CpG dinucleotide and human genetic disease. *Human genetics*, *78*(2), 151-155.

Cusanovich, D. A., Daza, R., Adey, A., Pliner, H. A., Christiansen, L., Gunderson, K. L., ... and Shendure, J. (2015). Multiplex single-cell profiling of chromatin accessibility by combinatorial cellular indexing. *Science*, *348*(6237), 910-914.

Daujat, S., Bauer, U. M., Shah, V., Turner, B., Berger, S., and Kouzarides, T. (2002). Crosstalk between CARM1 methylation and CBP acetylation on histone H3. *Current Biology*, *12*(24), 2090-2097.

David, C. J., and Manley, J. L. (2008). The search for alternative splicing regulators: new approaches offer a path to a splicing code. *Genes and development*, *22*(3), 279-285.

Davidson, E. H., and Erwin, D. H. (2006). Gene regulatory networks and the evolution of animal body plans. *Science*, *311*(5762), 796-800.

De Jong, G. (1990). Quantitative genetics of reaction norms. *Journal of Evolutionary Biology*, *3*(5-6), 447-468.

De Robertis, E. M. (2006). Spemann's organizer and self-regulation in amphibian embryos. *Nature Reviews Molecular Cell Biology*, *7*(4), 296-302.

Derrien, T., Guigó, R., and Johnson, R. (2011). The long non-coding RNAs: a new (p) layer in the “dark matter”. *Frontiers in genetics*, *2*.

Dickins, T. E., and Rahman, Q. (2012). The extended evolutionary synthesis and the role of soft inheritance in evolution. *Proceedings of the Royal Society of London B: Biological Sciences*, rspb20120273.

Dieterich, C., Clifton, S. W., Schuster, L. N., Chinwalla, A., Delehaunty, K., Dinkelacker, I., ... and Sommer, R. J. (2008). The Pristionchus pacificus genome provides a unique perspective on nematode lifestyle and parasitism. *Nature genetics*, *40*(10), 1193-1198.

Dodge, J. E., Ramsahoye, B. H., Wo, Z. G., Okano, M., and Li, E. (2002). De novo methylation of MMLV provirus in embryonic stem cells: CpG versus non-CpG methylation. *Gene*, *289*(1), 41-48.

Dozmorov, M. G., Giles, C. B., Koelsch, K. A., and Wren, J. D. (2013). Systematic classification of non-coding RNAs by epigenomic similarity. *BMC bioinformatics*, *14*(Suppl 14), S2.

Duncan, E. M., Muratore-Schroeder, T. L., Cook, R. G., Garcia, B. A., Shabanowitz, J., Hunt, D. F., and Allis, C. D. (2008). Cathepsin L proteolytically processes histone H3 during mouse embryonic stem cell differentiation. *Cell*, *135*(2), 284-294.

Egger, G., Liang, G., Aparicio, A., and Jones, P. A. (2004). Epigenetics in human disease and prospects for epigenetic therapy. *Nature*, *429*(6990), 457-463.

Fatica, A., and Bozzoni, I. (2014). Long non-coding RNAs: new players in cell differentiation and development. *Nature Reviews Genetics*, *15*(1), 7-21.

Feinberg, A. P. (2007). Phenotypic plasticity and the epigenetics of human disease. *Nature*, *447*(7143), 433-440.

Feinberg, A. P., and Irizarry, R. A. (2010). Stochastic epigenetic variation as a driving force of development, evolutionary adaptation, and disease. *Proceedings of the National Academy of Sciences*, *107*(suppl 1), 1757-1764.

Feinberg, A. P., and Tycko, B. (2004). The history of cancer epigenetics. *Nature Reviews Cancer*, *4*(2), 143-153.

Feinberg, A. P., and Vogelstein, B. (1983). Hypomethylation distinguishes genes of some human cancers from their normal counterparts. *Nature*, *301*(5895), 89-92.

Félix, M. A., and Wagner, A. (2008). Robustness and evolution: concepts, insights and challenges from a developmental model system. *Heredity*, *100*(2), 132-140.

Fielenbach, N., and Antebi, A. (2008). C. elegans dauer formation and the molecular basis of plasticity. *Genes and development*, *22*(16), 2149-2165.

Fraga, M. F., Ballestar, E., Villar-Garea, A., Boix-Chornet, M., Espada, J., Schotta, G., ... and Esteller, M. (2005). Loss of acetylation at Lys16 and trimethylation at Lys20 of histone H4 is a common hallmark of human cancer. *Nature genetics*, *37*(4), 391-400.

Fu, X., Ravindranath, L., Tran, N., Petrovics, G., and Srivastava, S. (2006). Regulation of apoptosis by a prostate-specific and prostate cancer-associated noncoding gene, PCGEM1. *DNA and cell biology*, *25*(3), 135-141.

Galloway, L. F. (2005). Maternal effects provide phenotypic adaptation to local environmental conditions. *New Phytologist*, *166*(1), 93-100.

Gilbert, S. F., and Epel, D. (2009). Ecological developmental biology: integrating epigenetics, medicine, and evolution.

Golden, J. W., and Riddle, D. L. (1984). A pheromone-induced developmental switch in Caenorhabditis elegans: Temperature-sensitive mutants reveal a wild-type temperature-dependent process. *Proceedings of the National Academy of Sciences*, *81*(3), 819-823.

Greer, E. L., Blanco, M. A., Gu, L., Sendinc, E., Liu, J., Aristizábal-Corrales, D., ... and Shi, Y. (2015). DNA Methylation on N 6-Adenine in C. elegans. *Cell*, *161*(4), 868-878.

Greer, E. L., Maures, T. J., Ucar, D., Hauswirth, A. G., Mancini, E., Lim, J. P., ... and Brunet, A. (2011). Transgenerational epigenetic inheritance of longevity in Caenorhabditis elegans. *Nature*, *479*(7373), 365-371.

Grewal, S. I., and Jia, S. (2007). Heterochromatin revisited. *Nature Reviews Genetics*, *8*(1), 35-46.

Guo, J. U., Agarwal, V., Guo, H., and Bartel, D. P. (2014). Expanded identification and characterization of mammalian circular RNAs. *Genome Biol*, *15*(409), 10-1186.

Guttman, M., and Rinn, J. L. (2012). Modular regulatory principles of large non-coding RNAs. *Nature*, *482*(7385), 339-346.

Hassett, G. P., Douglas, J., and Mancini, M. (2004). *U.S. Patent No. 6,807,558*. Washington, DC: U.S. Patent and Trademark Office.

He, K., and Wang, P. (2015). Unregulated long non-coding RNA-AK058003 promotes the proliferation, invasion and metastasis of breast cancer by regulating the expression levels of the γ-synuclein gene. *Experimental and Therapeutic Medicine*, *9*(5), 1727-1732.

He, Y., Cui, Y., Wang, W., Gu, J., Guo, S., Ma, K., and Luo, X. (2011). Hypomethylation of the hsa-miR-191 locus causes high expression of hsa-mir-191 and promotes the epithelial-to-mesenchymal transition in hepatocellular carcinoma. *Neoplasia*, *13*(9), 841-IN23.

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

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

Hirschmann, H. (1951). Über das Vorkommen zweier Mundhöhlentypen bei Diplogaster lheritieri Maupas und Diplogaster biformis n. sp. und die Entstehung dieser hermaphroditischen Art aus Diplogaster lheritieri. *Zool Jahrb*, *80*, 132-170.

Hodges, E., Smith, A. D., Kendall, J., Xuan, Z., Ravi, K., Rooks, M., ... and Hicks, J. B. (2009). High definition profiling of mammalian DNA methylation by array capture and single molecule bisulfite sequencing. *Genome research*, *19*(9), 1593-1605.

Holoch, D., and Moazed, D. (2015). RNA-mediated epigenetic regulation of gene expression. *Nature Reviews Genetics*.

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

Hoss, A. G., Kartha, V. K., Dong, X., Latourelle, J. C., Dumitriu, A., Hadzi, T. C., ... and Myers, R. H. (2014). MicroRNAs located in the Hox gene clusters are implicated in huntington's disease pathogenesis. *PLoS Genet*, *10*(2), e1004188.

Hu, X., Lu, X., Liu, R., Ai, N., Cao, Z., Li, Y., ... and Chen, R. (2014). Histone cross-talk connects protein phosphatase 1α (PP1α) and histone deacetylase (HDAC) pathways to regulate the functional transition of bromodomain-containing 4 (BRD4) for inducible gene expression. *Journal of Biological Chemistry*, *289*(33), 23154-23167.

Iyer, L. M., Abhiman, S., and Aravind, L. (2010). Natural history of eukaryotic DNA methylation systems. *Progress in molecular biology and translational science*, *101*, 25-104.

Jablonka, E. (2013). Epigenetic inheritance and plasticity: the responsive germline. *Progress in biophysics and molecular biology*, *111*(2), 99-107.

Jablonka, E., and Lamb, M. J. (1998). Epigenetic inheritance in evolution. *Journal of Evolutionary Biology*, *11*(2), 159-183.

Jablonka, E., and Lamb, M. J. (1999). *Epigenetic inheritance and evolution: the Lamarckian dimension*. Oxford University Press.

Jablonka, E., and Lamb, M. J. (2005). Evolution in four dimensions: Genetic. *Epigenetic, Behav*.

Jablonka, E., and Raz, G. (2009). Transgenerational epigenetic inheritance: prevalence, mechanisms, and implications for the study of heredity and evolution. *The Quarterly review of biology*, *84*(2), 131-176.

Jaenisch, R., and Bird, A. (2003). Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nature genetics*, *33*, 245-254.

Jeck, W. R., Sorrentino, J. A., Wang, K., Slevin, M. K., Burd, C. E., Liu, J., ... and Sharpless, N. E. (2013). Circular RNAs are abundant, conserved, and associated with ALU repeats. *Rna*, *19*(2), 141-157.

Jenner, R. A., and Wills, M. A. (2007). The choice of model organisms in evo–devo. *Nature Reviews Genetics*, *8*(4), 311-314.

Johnson, C. L., and Spence, A. M. (2011). Epigenetic licensing of germline gene expression by maternal RNA in C. elegans. *Science*, *333*(6047), 1311-1314.

Johnson, L. J., and Tricker, P. J. (2010). Epigenomic plasticity within populations: its evolutionary significance and potential. *Heredity*, *105*(1), 113-121.

Jones, P. A., and Baylin, S. B. (2002). The fundamental role of epigenetic events in cancer. *Nature reviews genetics*, *3*(6), 415-428.

Jorgensen, E. M., and Mango, S. E. (2002). The art and design of genetic screens: Caenorhabditis elegans. *Nature Reviews Genetics*, *3*(5), 356-369.

Jung, C. H., Hansen, M. A., Makunin, I. V., Korbie, D. J., and Mattick, J. S. (2010). Identification of novel non-coding RNAs using profiles of short sequence reads from next generation sequencing data. *BMC genomics*, *11*(1), 77.

Kalisz, S., and Purugganan, M. D. (2004). Epialleles via DNA methylation: consequences for plant evolution. *Trends in Ecology and Evolution*, *19*(6), 309-314.

Kan, J. Y., Wu, D. C., Yu, F. J., Wu, C. Y., Ho, Y. W., Chiu, Y. J., ... and Kuo, P. L. (2015). Chemokine (C-C Motif) Ligand 5 is Involved in Tumor-Associated Dendritic Cell-Mediated Colon Cancer Progression Through Non-Coding RNA MALAT-1. *Journal of cellular physiology*, *230*(8), 1883-1894.

Kanhere, A., and Jenner, R. G. (2012). Noncoding RNA localisation mechanisms in chromatin regulation. *Silence*, *3*(1), 2-2.

Kannan, S., Chernikova, D., Rogozin, I. B., Poliakov, E., Managadze, D., Koonin, E. V., and Milanesi, L. (2015). Transposable element insertions in long intergenic non-coding RNA genes. *Frontiers in Bioengineering and Biotechnology*, *3*, 71.

Kanzaki, N., Ragsdale, E. J., Herrmann, M., Mayer, W. E., and Sommer, R. J. (2012). Description of three Pristionchus species (Nematoda: Diplogastridae) from Japan that form a cryptic species complex with the model organism P. pacificus. *Zoological science*, *29*(6), 403-417.

Kanzaki, N., Ragsdale, E. J., Herrmann, M., Röseler, W., and Sommer, R. J. (2013a). Pristionchus bucculentus n. sp.(Rhabditida: Diplogastridae) isolated from a shining mushroom beetle (Coleoptera: Scaphidiidae) in Hokkaido, Japan. *Journal of nematology*, *45*(1), 78.

Kanzaki, N., Ragsdale, E. J., Herrmann, M., Röseler, W., and Sommer, R. J. (2013b). Two new species of Pristionchus (Nematoda: Diplogastridae) support the biogeographic importance of Japan for the evolution of the genus Pristionchus and the model system P. pacificus. *Zoological science*, *30*(8), 680-692.

Kapranov, P., Cheng, J., Dike, S., Nix, D. A., Duttagupta, R., Willingham, A. T., ... and Gingeras, T. R. (2007). RNA maps reveal new RNA classes and a possible function for pervasive transcription. *Science*, *316*(5830), 1484-1488.

Kaufman, E. J., and Miska, E. A. (2010, September). The microRNAs of Caenorhabditis elegans. In *Seminars in cell and developmental biology* (Vol. 21, No. 7, pp. 728-737). Academic Press.

Kelly, S. A., Panhuis, T. M., and Stoehr, A. M. (2012). Phenotypic plasticity: molecular mechanisms and adaptive significance. *Comprehensive Physiology*.

Khalil, A. M., Guttman, M., Huarte, M., Garber, M., Raj, A., Morales, D. R., ... and Rinn, J. L. (2009). Many human large intergenic noncoding RNAs associate with chromatin-modifying complexes and affect gene expression. *Proceedings of the National Academy of Sciences*, *106*(28), 11667-11672.

Kirschner, M., and Gerhart, J. (1998). Comment on “epigenetic inheritance in evolution”. *Journal of Evolutionary Biology*, *11*(2), 213-217.

Kishore, S., and Stamm, S. (2006). The snoRNA HBII-52 regulates alternative splicing of the serotonin receptor 2C. *Science*, *311*(5758), 230-232.

Kolasinska-Zwierz, P., Down, T., Latorre, I., Liu, T., Liu, X. S., and Ahringer, J. (2009). Differential chromatin marking of introns and expressed exons by H3K36me3. *Nature genetics*, *41*(3), 376-381.

Kornberg, R. D. (1977). Structure of chromatin. *Annual review of biochemistry*, *46*(1), 931-954.

Kota, S. K., Chowdhury, D. R., Rao, L. K., Padmalatha, V., Singh, L., and Bhadra, U. (2014). Uncoupling of X-linked gene silencing from XIST binding by DICER1 and chromatin modulation on human inactive X chromosome. *Chromosoma*, 1-14.

Kuijper, B., and Hoyle, R. B. (2015). When to rely on maternal effects and when on phenotypic plasticity?. *Evolution*, *69*(4), 950-968.

Kuroyanagi, H., Kimura, T., Wada, K., Hisamoto, N., Matsumoto, K., and Hagiwara, M. (2000). SPK-1, a C. elegans SR protein kinase homologue, is essential for embryogenesis and required for germline development. *Mechanisms of development*, *99*(1), 51-64.

Labouesse, M., and Mango, S. E. (1999). Patterning the C. elegans embryo: moving beyond the cell lineage. *Trends in Genetics*, *15*(8), 307-313.

Lai, M. C., Yang, Z., Zhou, L., Zhu, Q. Q., Xie, H. Y., Zhang, F., ... and Zheng, S. S. (2012). Long non-coding RNA MALAT-1 overexpression predicts tumor recurrence of hepatocellular carcinoma after liver transplantation. *Medical oncology*, *29*(3), 1810-1816.

Lee, J. S., and Shilatifard, A. (2007). A site to remember: H3K36 methylation a mark for histone deacetylation. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, *618*(1), 130-134.

Lee, J. S., Shukla, A., Schneider, J., Swanson, S. K., Washburn, M. P., Florens, L., ... and Shilatifard, A. (2007). Histone crosstalk between H2B

monoubiquitination and H3 methylation mediated by COMPASS. *Cell*, *131*(6), 1084-1096.

Lee, J. T., and Bartolomei, M. S. (2013). X-inactivation, imprinting, and long noncoding RNAs in health and disease. *Cell*, *152*(6), 1308-1323.
Lee, Y., and Rio, D. C. (2015). Mechanisms and Regulation of Alternative Pre-mRNA Splicing. *Annual review of biochemistry*, (0).

Lehner, B., Crombie, C., Tischler, J., Fortunato, A., and Fraser, A. G. (2006). Systematic mapping of genetic interactions in Caenorhabditis elegans identifies common modifiers of diverse signaling pathways. *Nature genetics*, *38*(8), 896-903.

Li, E., Bestor, T. H., and Jaenisch, R. (1992). Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. *Cell*, *69*(6), 915-926.

Li, L., Dang, Q., Xie, H., Yang, Z., He, D., Liang, L., ... and Chang, C. (2015). Infiltrating mast cells enhance prostate cancer invasion via altering LncRNA-HOTAIR/PRC2-androgen receptor (AR)-MMP9 signals and increased stem/progenitor cell population. *Oncotarget*.

Li, S., and Shogren-Knaak, M. A. (2008). Cross-talk between histone H3 tails produces cooperative nucleosome acetylation. *Proceedings of the National Academy of Sciences*, *105*(47), 18243-18248.

Li, Z., Zhao, X., Zhou, Y., Liu, Y., Zhou, Q., Ye, H., ... and Chen, R. (2015). The long non-coding RNA HOTTIP promotes progression and gemcitabine resistance by regulating HOXA13 in pancreatic cancer. *Journal of translational medicine*, *13*(1), 84.

Liefting, M., Hoffmann, A. A., and Ellers, J. (2009). Plasticity versus environmental canalization: population differences in thermal responses along a latitudinal gradient in Drosophila serrata. *Evolution*, *63*(8), 1954-1963.

Lin, L., Chen, L., Wang, H., Wei, X., Fu, X., Zhang, J., ... and Yu, C. (2006). Adenovirus-mediated transfer of siRNA against Runx2/Cbfa1 inhibits the formation of heterotopic ossification in animal model. *Biochemical and biophysical research communications*, *349*(2), 564-572.

Luger, K., Rechsteiner, T. J., Flaus, A. J., Waye, M. M., and Richmond, T. J. (1997). Characterization of nucleosome core particles containing histone proteins made in bacteria. *Journal of molecular biology*, *272*(3), 301-311.

Maile, R., Jones, S., Pan, Y., Zhou, H., Jaspers, I., Peden, D. B., ... and Noah, T. L. (2015). Association between early airway damage-associated molecular

patterns and subsequent bacterial infection in patients with inhalational and burn injury. *American Journal of Physiology-Lung Cellular and Molecular Physiology*, *308*(9), L855-L860.

Marquardt, S., Escalante-Chong, R., Pho, N., Wang, J., Churchman, L. S., Springer, M., and Buratowski, S. (2014). A chromatin-based mechanism for limiting divergent noncoding transcription. *Cell*, *157*(7), 1712-1723.

Mather, K. (1953). Genetical control of stability in development. *Heredity*, *7*(3), 297-336.

Mather, K. (1973). Genetic structure of populations. *Genetic structure of populations.*, (Ed. 1).

Mattick, J. S., and Makunin, I. V. (2006). Non-coding RNA. *Human molecular genetics*, *15*(suppl 1), R17-R29.

Memczak, S., Jens, M., Elefsinioti, A., Torti, F., Krueger, J., Rybak, A., ... and Rajewsky, N. (2013). Circular RNAs are a large class of animal RNAs with regulatory potency. *Nature*, *495*(7441), 333-338.

Mercer, T. R., and Mattick, J. S. (2013). Structure and function of long noncoding RNAs in epigenetic regulation. *Nature structural and molecular biology*, *20*(3), 300-307.

Mercer, T. R., Dinger, M. E., and Mattick, J. S. (2009). Long non-coding RNAs: insights into functions. *Nature Reviews Genetics*, *10*(3), 155-159.
Meyer, B. J. (2000). Sex in the worm: counting and compensating X-chromosome dose. *Trends in Genetics*, *16*(6), 247-253.

Misteli, T., Gunjan, A., Hock, R., Bustin, M., and Brown, D. T. (2000). Dynamic binding of histone H1 to chromatin in living cells. *Nature*, *408*(6814), 877-881.

Mitchell, A. C., Pothuka, V., and Akbarian, S. (2015, May). Exploring the Genome in Three Dimensions in the Prefrontal Cortex: Discovering the Functional Relevance of Non-Coding Regions of the Genome. In *Biological Psychiatry* (Vol. 77, No. 9, pp. 414S-414S). 360 Park Ave South, New York, NY 10010-1710 USA: ELSEVIER SCIENCE INC.

Miura, T. (2005). Developmental regulation of caste-specific characters in social-insect polyphenism. *Evolution and development*, *7*(2), 122-129.

Moazed, D. (2009). Small RNAs in transcriptional gene silencing and genome defence. *Nature*, *457*(7228), 413-420.

Mousseau, T. A., and Fox, C. W. (1998). The adaptive significance of maternal effects. *Trends in Ecology and Evolution*, *13*(10), 403-407.

Mousseau, T. A., and Fox, C. W. (1998). The adaptive significance of maternal effects. *Trends in Ecology and Evolution*, *13*(10), 403-407.

Muegge, K. (2003). Modifications of histone cores and tails in V (D) J recombination. *Genome Biol*, *4*, 211.

Nartey, T., Moran, H., Marin, T., Arcaro, K. F., Anderton, D. L., Etkind, P., ... and Pogo, B. G. (2014). Human Mammary Tumor Virus (HMTV) sequences in human milk. *Infect Agent Cancer*, *9*, 20.

Newlon, A. W., Yund, P. O., and Stewart-Savage, J. (2003). Phenotypic plasticity of reproductive effort in a colonial ascidian, Botryllus schlosseri. *Journal of Experimental Zoology Part A: Comparative Experimental Biology*, *297*(2), 180-188.

Nguyen, A. L., Gentilello, A. S., Balboula, A. Z., Shrivastava, V., Ohring, J., and Schindler, K. (2014). Phosphorylation of threonine 3 on histone H3 by haspin kinase is required for meiosis I in mouse oocytes. *Journal of cell science*, *127*(23), 5066-5078.

Nijhout, H. F. (1999). Control Mechanisms of Polyphenic Development in Insects In polyphenic development, environmental factors alter some aspects of development in an orderly and predictable way. *Bioscience*, *49*(3), 181-192.

Nijhout, H. F. (2003). Development and evolution of adaptive polyphenisms. *Evolution and development*, *5*(1), 9-18.

Nilsen, T. W., and Graveley, B. R. (2010). Expansion of the eukaryotic proteome by alternative splicing. *Nature*, *463*(7280), 457-463.

Noh, K. M., Maze, I., Zhao, D., Xiang, B., Wenderski, W., Lewis, P. W., ... and Allis, C. D. (2014). ATRX tolerates activity-dependent histone H3 methyl/phos switching to maintain repetitive element silencing in neurons. *Proceedings of the National Academy of Sciences*, 201411258.

Noyer-Weidner, M., and Trautner, T. A. (1993). Methylation of DNA in prokaryotes. In *DNA Methylation* (pp. 39-108). Birkhäuser Basel.

Nozawa, R. S., Nagao, K., Igami, K. T., Shibata, S., Shirai, N., Nozaki, N., ... and Obuse, C. (2013). Human inactive X chromosome is compacted through a PRC2-independent SMCHD1-HBiX1 pathway. *Nature structural and molecular biology*, *20*(5), 566-573.

Nussey, D. H., Kruuk, L. E., Morris, A., and Clutton-Brock, T. H. (2007). Environmental conditions in early life influence ageing rates in a wild population of red deer. *Current Biology*, *17*(23), R1000-R1001.

Nüsslein-Volhard, C., Frohnhofer, H. G., and Lehmann, R. (1987). Determination of anteroposterior polarity in Drosophila. *Science*, *238*(4834), 1675-1681.

Nüsslein-Volhard, C., Lohs-Schardin, M., Sander, K., and Cremer, C. (1980). A dorso-ventral shift of embryonic primordia in a new maternal-effect mutant of Drosophila. *Nature*, *283*(5746), 474-476.

Nylin, S., and Gotthard, K. (1998). Plasticity in life-history traits. *Annual review of entomology*, *43*(1), 63-83.

Ogawa, A., Bento, G., Bartelmes, G., Dieterich, C., and Sommer, R. J. (2011). Pristionchus pacificus daf-16 is essential for dauer formation but dispensable for mouth form dimorphism. *Development*, *138*(7), 1281-1284.

Ørom, U. A., Derrien, T., Guigo, R., and Shiekhattar, R. (2011, April). Long noncoding RNAs as enhancers of gene expression. In *Cold Spring Harbor symposia on quantitative biology* (pp. sqb-2010). Cold Spring Harbor Laboratory Press.

Pan, Q., Shai, O., Lee, L. J., Frey, B. J., and Blencowe, B. J. (2008). Deep surveying of alternative splicing complexity in the human transcriptome by high-throughput sequencing. *Nature genetics*, *40*(12), 1413-1415.

Pannucci, J. A., Haas, E. S., Hall, T. A., Harris, J. K., and Brown, J. W. (1999). RNase P RNAs from some Archaea are catalytically active. *Proceedings of the National Academy of Sciences*, *96*(14), 7803-7808.

Pelch, K. E., Tokar, E. J., Merrick, B. A., and Waalkes, M. P. (2015). Differential DNA methylation profile of key genes in malignant prostate epithelial cells transformed by inorganic arsenic or cadmium. *Toxicology and applied pharmacology*.

Penny, G. D., Kay, G. F., Sheardown, S. A., Rastan, S., and Brockdorff, N. (1996). Requirement for Xist in X chromosome inactivation. *Nature*, *379*(6561), 131-137.

Perreault, A., Lemieux, C., and Bachand, F. (2007). Regulation of the nuclear poly (A)-binding protein by arginine methylation in fission yeast. *Journal of Biological Chemistry*, *282*(10), 7552-7562.

Pfennig, D. (1990). The adaptive significance of an environmentally-cued developmental switch in an anuran tadpole. *Oecologia*, *85*(1), 101-107.

Pigliucci, M., Schlichting, C. D., Jones, C. S., and Schwenk, K. (1996). Developmental reaction norms: the interactions among allometry, ontogeny and plasticity. *Plant Species Biology*, *11*(1), 69-85.

Pires-daSilva, A. (2006) *Pristionchus pacificus* genetic protocols, WormBook, ed.

Pradeepa, M. M., Grimes, G. R., Taylor, G. C., Sutherland, H. G., and Bickmore, W. A. (2014). Psip1/Ledgf p75 restrains Hox gene expression by recruiting both trithorax and polycomb group proteins. *Nucleic acids research*, *42*(14), 9021-9032.

Price, T. D., Qvarnström, A., and Irwin, D. E. (2003). The role of phenotypic plasticity in driving genetic evolution. *Proceedings of the Royal Society of London B: Biological Sciences*, *270*(1523), 1433-1440

Qiu, J. (2006). Epigenetics: unfinished symphony. *Nature*, *441*(7090), 143-145.

Qureshi, I. A., and Mehler, M. F. (2014). Epigenetic Mechanisms Underlying the Pathogenesis of Neurogenetic Diseases. *Neurotherapeutics*, *11*(4), 708-720.
Raff, R. A. (1996). The shape of evolutionary developmental biology.

Raff, R. A., and Wray, G. A. (1989). Heterochrony: developmental mechanisms and evolutionary results. *Journal of Evolutionary Biology*, *2*(6), 409-434.

Ragsdale, E. J., Kanzaki, N., and Sommer, R. J. (2014). Levipalatum texanum n. gen., n. sp.(Nematoda: Diplogastridae), an androdioecious species from the south-eastern USA. *Nematology*, *16*(6), 695-709.

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

Ragsdale, E. J., Kanzaki, N., Röseler, W., Herrmann, M., and Sommer, R. J. (2013b). Three new species of Pristionchus (Nematoda: Diplogastridae) show morphological divergence through evolutionary intermediates of a novel feeding-structure polymorphism. *Zoological Journal of the Linnean Society*, *168*(4), 671-698.

Ramakrishnan, V., Finch, J. T., Graziano, V., Lee, P. L., and Sweet, R. M. (1993). Crystal structure of globular domain of histone H5 and its implications for nucleosome binding. *nature*, *362*(6417), 219-223.

Rambaldi, D., and Ciccarelli, F. D. (2009). FancyGene: dynamic visualization of gene structures and protein domain architectures on genomic loci. *Bioinformatics*, *25*(17), 2281-2282.

Rapp, R. A., and Wendel, J. F. (2005). Epigenetics and plant evolution. *New Phytologist*, *168*(1), 81-91.

Rechavi, O., Minevich, G., and Hobert, O. (2011). Transgenerational inheritance of an acquired small RNA-based antiviral response in C. elegans. *Cell*, *147*(6), 1248-1256.

Resendiz, M., Chen, Y., Öztürk, N. C., and Zhou, F. C. (2013). Epigenetic medicine and fetal alcohol spectrum disorders. *Epigenomics*, *5*(1), 73-86.

Richards, C. L., Bossdorf, O., and Pigliucci, M. (2010). What role does heritable epigenetic variation play in phenotypic evolution?. *BioScience*, *60*(3), 232-237.

Riggs, A. D. (1975). X inactivation, differentiation, and DNA methylation. *Cytogenetic and Genome Research*, *14*(1), 9-25.

Rinn, J. L., and Chang, H. Y. (2012). Genome regulation by long noncoding RNAs. *Annual review of biochemistry*, *81*.

Robertson, K. D., Manns, A., Swinnen, L. J., Zong, J. C., Gulley, M. L., and Ambinder, R. F. (1996). CpG methylation of the major Epstein-Barr virus latency promoter in. *Blood*, *88*(8), 3129-3136.

Rodenas-Ruano, A., Chávez, A. E., Cossio, M. J., Castillo, P. E., and Zukin, R. S. (2012). REST-dependent epigenetic remodeling promotes the developmental switch in synaptic NMDA receptors. *Nature neuroscience*, *15*(10), 1382-1390.

Roskam, J. C., and Brakefield, P. M. (1999). Seasonal polyphenism in Bicyclus (Lepidoptera: Satyridae) butterflies: different climates need different cues. *Biological Journal of the Linnean Society*, *66*(3), 345-356.

Roudier, F., Ahmed, I., Bérard, C., Sarazin, A., Mary-Huard, T., Cortijo, S., ... and Colot, V. (2011). Integrative epigenomic mapping defines four main chromatin states in Arabidopsis. *The EMBO journal*, *30*(10), 1928-1938.

Sado, T., Wang, Z., Sasaki, H., and Li, E. (2001). Regulation of imprinted X-chromosome inactivation in mice by Tsix. *Development*, *128*(8), 1275-1286.

Salmena, L., Poliseno, L., Tay, Y., Kats, L., and Pandolfi, P. P. (2011). A ceRNA hypothesis: the Rosetta Stone of a hidden RNA language?. *Cell*, *146*(3), 353-358.

Salzman, J., Gawad, C., Wang, P. L., Lacayo, N., and Brown, P. O. (2012). Circular RNAs are the predominant transcript isoform from hundreds of human genes in diverse cell types. *PloS one*, *7*(2), e30733.

Sanders, S. L., Portoso, M., Mata, J., Bähler, J., Allshire, R. C., and Kouzarides, T. (2004). Methylation of histone H4 lysine 20 controls recruitment of Crb2 to sites of DNA damage. *Cell*, *119*(5), 603-614.

Scharloo, W. (1970). Stabilizing and disruptive selection on a mutant character in Drosophila. III. Polymorphism caused by a developmental switch mechanism. *Genetics*, *65*(4), 693.

Schier, A. F. (2007). The maternal-zygotic transition: death and birth of RNAs. *Science*, *316*(5823), 406-407.

Schlager, B., Wang, X., Braach, G., and Sommer, R. J. (2009). Molecular cloning of a dominant roller mutant and establishment of DNA-mediated transformation in the nematode Pristionchus pacificus. *Genesis*, *47*(5), 300-304.

Schlichting, C. D., and Wund, M. A. (2014). Phenotypic plasticity and epigenetic marking: an assessment of evidence for genetic accommodation. *Evolution*, *68*(3), 656-672.

Schmalhausen, I. I. (1949). Factors of evolution: the theory of stabilizing selection.

Schmitz, R. J., Schultz, M. D., Lewsey, M. G., O'Malley, R. C., Urich, M. A., Libiger, O., ... and Ecker, J. R. (2011). Transgenerational epigenetic instability is a source of novel methylation variants. *Science*, *334*(6054), 369-373.

Schoeftner, S., Sengupta, A. K., Kubicek, S., Mechtler, K., Spahn, L., Koseki, H., ... and Wutz, A. (2006). Recruitment of PRC1 function at the initiation of X inactivation independent of PRC2 and silencing. *The EMBO journal*, *25*(13), 3110-3122.

Schüpbach, T., and Wieschaus, E. (1986). Germline autonomy of maternal-effect mutations altering the embryonic body pattern of Drosophila. *Developmental biology*, *113*(2), 443-448.

Schuster, L. N., and Sommer, R. J. (2012). Expressional and functional variation of horizontally acquired cellulases in the nematode Pristionchus pacificus. *Gene*, *506*(2), 274-282.

Schwämmle, V., Aspalter, C. M., Sidoli, S., and Jensen, O. N. (2014). Large scale analysis of co-existing post-translational modifications in histone tails reveals global fine structure of cross-talk. *Molecular and Cellular Proteomics*, *13*(7), 1855-1865.

Schwartz, S., Meshorer, E., and Ast, G. (2009). Chromatin organization marks exon-intron structure. *Nature structural and molecular biology*, *16*(9), 990-995.

Sequencing Consortium. (1998). Genome sequence of the nematode *C. elegans*: A platform for investigating biology. *Science*, *282*, 2012-2018.

Serobyan, V., Ragsdale, E. J., Müller, M. R., and Sommer, R. J. (2013). Feeding plasticity in the nematode Pristionchus pacificus is influenced by sex and social context and is linked to developmental speed. *Evolution and development*, *15*(3), 161-170.

Shahbazian, M. D., Young, J. I., Yuva-Paylor, L. A., Spencer, C. M., Antalffy, B. A., Noebels, J. L., ... and Zoghbi, H. Y. (2002). Mice with truncated MeCP2 recapitulate many Rett syndrome features and display hyperacetylation of histone H3. *Neuron*, *35*(2), 243-254.

Shea, N., Pen, I., and Uller, T. (2011). Three epigenetic information channels and their different roles in evolution. *Journal of evolutionary biology*, *24*(6), 1178-1187.

Simpson, V. J., Johnson, T. E., and Hammen, R. F. (1986). Caenorhabditis elegans DNA does not contain 5-methylcytosine at any time during development or aging. *Nucleic acids research*, *14*(16), 6711-6719.

Smith, G., and Ritchie, M. G. (2013). How might epigenetics contribute to ecological speciation?. *Current Zoology*.

Sommer, R. J. (2005). Evolution of development in nematodes related to *C. elegans*.

Sommer, R. J. (2006). New inroads to development. *Development*, *133*(13), 2451-2453.

Sommer, R. J. (2009). The future of evo–devo: model systems and evolutionary theory. *Nature Reviews Genetics*, *10*(6), 416-422.

Sommer, R. J., and McGaughran, A. (2013). The nematode Pristionchus pacificus as a model system for integrative studies in evolutionary biology. *Molecular ecology*, *22*(9), 2380-2393.

Sommer, R. J., and Sternberg, P. W. (1996). Evolution of nematode vulval fate patterning. *Developmental biology*, *173*(2), 396-407.

Sommer, R., Carta, L. K., Kim, S. Y., and Sternberg, P. W. (1996). Morphological, genetic and molecular description of Pristionchus pacificus sp.

n.(Nematoda: Neodiplogasteridae). *Fundamental and applied Nematology*, *19*, 511-522.

Spemann, H. (1962). *Embryonic development and induction*. Hafner Publishing Company.

Spies, N., Nielsen, C. B., Padgett, R. A., and Burge, C. B. (2009). Biased chromatin signatures around polyadenylation sites and exons. *Molecular cell*, *36*(2), 245-254.

Srinivasan, J., Sinz, W., Lanz, C., Brand, A., Nandakumar, R., Raddatz, G., ... and Sommer, R. J. (2002). A bacterial artificial chromosome-based genetic linkage map of the nematode Pristionchus pacificus. *Genetics*, *162*(1), 129-134.

Sternberg, P. W. (2005). Vulval development. *WormBook*, *25*, 1-28.
Stibor, H. (1992). Predator induced life-history shifts in a freshwater cladoceran. *Oecologia*, *92*(2), 162-165.

Strome, S., Kelly, W. G., Ercan, S., and Lieb, J. D. (2014). Regulation of the X Chromosomes in Caenorhabditis elegans. *Cold Spring Harbor perspectives in biology*, *6*(3), a018366.

Suganuma, T., and Workman, J. L. (2008). Crosstalk among histone modifications. *Cell*, *135*(4), 604-607.

Sulston, J. E., and Horvitz, H. R. (1977). Post-embryonic cell lineages of the nematode, Caenorhabditis elegans. *Developmental biology*, *56*(1), 110-156.

Sulston, J. E., and Horvitz, H. R. (1981). Abnormal cell lineages in mutants of the nematode Caenorhabditis elegans. *Developmental biology*, *82*(1), 41-55.

Suzuki, M. M., and Bird, A. (2008). DNA methylation landscapes: provocative insights from epigenomics. *Nature Reviews Genetics*, *9*(6), 465-476.

Suzuki, Y., and Nijhout, H. F. (2006). Evolution of a polyphenism by genetic accommodation. *Science*, *311*(5761), 650-652.

Tabuchi, T. M., Rechtsteiner, A., Strome, S., and Hagstrom, K. A. (2014). Opposing activities of DRM and MES-4 tune gene expression and X-chromosome repression in Caenorhabditis elegans germ cells. *G3: Genes| Genomes| Genetics*, *4*(1), 143-153.

Thoday, J. M. (1964). *Genetics and the integration of reproductive systems*. na.

Thornton, J. L., Westfield, G. H., Takahashi, Y. H., Cook, M., Gao, X., Woodfin, A. R., ... and Shilatifard, A. (2014). Context dependency of Set1/COMPASS-

mediated histone H3 Lys4 trimethylation. *Genes and development*, *28*(2), 115-120.

Tian, H., Schlager, B., Xiao, H., and Sommer, R. J. (2008). Wnt signaling induces vulva development in the nematode Pristionchus pacificus. *Current Biology*, *18*(2), 142-146.

Tjeertes, J. V., Miller, K. M., and Jackson, S. P. (2009). Screen for DNA-damage-responsive histone modifications identifies H3K9Ac and H3K56Ac in human cells. *The EMBO journal*, *28*(13), 1878-1889.

Tollrian, R., and Harvell, C. D. (1999). *The ecology and evolution of inducible defenses*. Princeton University Press.

Trapnell, C., Roberts, A., Goff, L., Pertea, G., Kim, D., Kelley, D. R., ... and Pachter, L. (2012). Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nature protocols*, *7*(3), 562-578.

True, J. R., and Haag, E. S. (2001). Developmental system drift and flexibility in evolutionary trajectories. *Evolution and development*, *3*(2), 109-119.

Tushir, J. S., and Akbarian, S. (2014). Chromatin-bound RNA and the neurobiology of psychiatric disease. *Neuroscience*, *264*, 131-141.

Uchida, H., Sasaki, K., Ma, L., and Ueda, H. (2010). Neuron-restrictive silencer factor causes epigenetic silencing of K v 4.3 gene after peripheral nerve injury. *Neuroscience*, *166*(1), 1-4.

Valena, S., and Moczek, A. P. (2012). Epigenetic mechanisms underlying developmental plasticity in horned beetles. *Genetics research international*, *2012*.

van Heesbeen, H. J., Mesman, S., Veenvliet, J. V., and Smidt, M. P. (2013). Epigenetic mechanisms in the development and maintenance of dopaminergic neurons. *Development*, *140*(6), 1159-1169.

Van Speybroeck, L. (2002). Philosophers and biologists exploring epigenetics. *Biology and Philosophy*, *17*(5), 743-746.

Varambally, S., Dhanasekaran, S. M., Zhou, M., Barrette, T. R., Kumar-Sinha, C., Sanda, M. G., ... and Chinnaiyan, A. M. (2002). The polycomb group protein EZH2 is involved in progression of prostate cancer. *nature*, *419*(6907), 624-629.

Via, S., and Lande, R. (1985). Genotype-environment interaction and the evolution of phenotypic plasticity. *Evolution*, 505-522.

Von Dassow, G., and Munro, E. (1999). Modularity in animal development and evolution: elements of a conceptual framework for EvoDevo. *The Journal of experimental zoology*, *285*(4), 307-325.

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

Waddington, C. H. (1942). Canalization of development and the inheritance of acquired characters. *Nature*, *150*(3811), 563-565.

Waddington, C. H. (1953). Genetic assimilation of an acquired character. *Evolution*, 118-126.

Walsh, C. P., Chaillet, J. R., and Bestor, T. H. (1998). Transcription of IAP endogenous retroviruses is constrained by cytosine methylation. *Nature genetics*, *20*(2), 116-117.

Wang, F., Li, L., Xu, H., Liu, Y., Yang, C., Cowley Jr, A. W., ... and Liang, M. (2014). Characteristics of Long Non-coding RNAs in the Brown Norway Rat and Alterations in the Dahl Salt-Sensitive Rat. *Scientific reports*, *4*.

Wang, X., and Sommer, R. J. (2011). Antagonism of LIN-17/Frizzled and LIN-18/Ryk in nematode vulva induction reveals evolutionary alterations in core developmental pathways. *PLoS-Biology*, *9*(7), 1487.

Weller, A. M., Mayer, W. E., Rae, R., and Sommer, R. J. (2010). Quantitative assessment of the nematode fauna present on Geotrupes dung beetles reveals species-rich communities with a heterogeneous distribution. *Journal of Parasitology*, *96*(3), 525-531.

West-Eberhard, M. J. (2003). *Developmental plasticity and evolution*. Oxford University Press.

Whitcomb, S. J., Fierz, B., McGinty, R. K., Holt, M., Ito, T., Muir, T. W., and Allis, C. D. (2012). Histone monoubiquitylation position determines specificity and direction of enzymatic cross-talk with histone methyltransferases Dot1L and PRC2. *Journal of Biological Chemistry*, *287*(28), 23718-23725.

White, J. G., Southgate, E., Thomson, J. N., and Brenner, S. (1986). The structure of the nervous system of the nematode Caenorhabditis elegans: the mind of a worm. *Phil. Trans. R. Soc. Lond*, *314*, 1-340.

Wilkins, A. S. (2002). *The evolution of developmental pathways*. Sunderland, Massachusetts, USA: Sinauer Associates Inc.

Witte, H., Moreno, E., Rödelsperger, C., Kim, J., Kim, J. S., Streit, A., and Sommer, R. J. (2015). Gene inactivation using the CRISPR/Cas9 system in the nematode Pristionchus pacificus. *Development genes and evolution*, *225*(1), 55-62.

Wolf, J. B., and Hager, R. (2006). A maternal-offspring coadaptation theory for the evolution of genomic imprinting. *PLoS Biol*, *4*(12), e380.

Wolf, S. F., Jolly, D. J., Lunnen, K. D., Friedmann, T., and Migeon, B. R. (1984). Methylation of the hypoxanthine phosphoribosyltransferase locus on the human X chromosome: implications for X-chromosome inactivation. *Proceedings of the National Academy of Sciences*, *81*(9), 2806-2810.

Wright, D. E., and Kao, C. F. (2015). (Ubi) quitin'the h2bit: recent insights into the roles of H2B ubiquitylation in DNA replication and transcription. *Epigenetics*, *10*(2), 122-126.

Wu, L., Lee, S. Y., Zhou, B., Nguyen, U. T., Muir, T. W., Tan, S., and Dou, Y. (2013). ASH2L regulates ubiquitylation signaling to MLL: trans-regulation of H3 K4 methylation in higher eukaryotes. *Molecular cell*, *49*(6), 1108-1120.

Wu, Y., Halverson, G., Basir, Z., Strawn, E., Yan, P., and Guo, S. W. (2005). Aberrant methylation at HOXA10 may be responsible for its aberrant expression in the endometrium of patients with endometriosis. *American journal of obstetrics and gynecology*, *193*(2), 371-380.

Yoon, J. H., Abdelmohsen, K., Srikantan, S., Yang, X., Martindale, J. L., De, S., ... and Gorospe, M. (2012). LincRNA-p21 suppresses target mRNA translation. *Molecular cell*, *47*(4), 648-655.

Yu, G., Bing, Y., Li, W., Xia, L., and Liu, Z. (2014). Hepatitis B virus inhibits the expression of CD82 through hypermethylation of its promoter in hepatoma cells. *Molecular medicine reports*, *10*(5), 2580-2586.

Zentner, G. E., and Henikoff, S. (2013). Regulation of nucleosome dynamics by histone modifications. *Nature structural and molecular biology*, *20*(3), 259-266.

Zhang, S., Banerjee, D., and Kuhn, J. R. (2011). Isolation and culture of larval cells from C. elegans. *PloS one*, *6*(4), e19505-e19505.

Zheng, M., Messerschmidt, D., Jungblut, B., and Sommer, R. J. (2005). Conservation and diversification of Wnt signaling function during the evolution of nematode vulva development. *Nature genetics*, *37*(3), 300-304.

Zhou, H. L., Luo, G., Wise, J. A., and Lou, H. (2014). Regulation of alternative splicing by local histone modifications: potential roles for RNA-guided mechanisms. *Nucleic acids research*, *42*(2), 701-713.

Acknowledgements

Foremost, I would like to thank my advisor, Prof. Dr. Ralf Sommer, for the continuous support of my PhD study and research. He patiently provided the vision, encouragement and advice necessary for me to proceed through my research. Being a supportive adviser, he always gave me the freedom to pursue independent work. Without his guidance and persistence this dissertation would not have been possible.

I would like to express my gratitude to my PhD defense committee members: Prof. Dr. Alfred Nordheim for evaluating this dissertation, Prof. Dr. Katharina Förster and Dr. Patrick Müller for their interest and feedbacks to my project and for being my examiners.

I am very thankful to all, current and past, members of the mouth-form team: Dr. Gilberto Bento, Manuela Kieninger, Dr. Erik Ragsdale, Dr. Joséphine Piffaretti, Bogdan Sieriebriennikov and Suryesh Namdeo. I was very fortunate to know you all; your friendship and assistance has meant more to me than I could ever express, and your support helped me to get through several complications during my work.

I would like thank Dr. Gilberto Bento for his support and care about me since my arrival, which allowed me to have the feeling that I am in my home country. Also, I would like to thank him for the great introduction to *P. pacificus* and its mouth-form.

A special thanks goes to Dr. Erik Ragsdale. It was really a fortunate to have him as a colleague, to share the lab-work in some projects, have fruitful discussion, and, most importantly his generous personality and support at the times of frustration made my days brighter. Erik, I enjoyed your company very much and hope to keep in touch always.

I am grateful to a colleague and a friend, Manuela Kieninger, who was helping me a lot and was very supportive not only in the lab but also outside of the lab.

I would like to acknowledge Dr. Melanie Mayer for critically reading the early

version of the thesis, beautifying the English of this dissertation and translating the summary into German.

I thank Dr. Adrian Streit for continuous advice and feedback to my project. Many, many thanks to Hanh, Gabi, Tobias, Walli, Metta, Dorothee, Heike and Sandra for their incredible amount of work, their invaluable tips and advice in various aspects of my work. Their professionalism has an inestimable impact on my thesis project!

I am also grateful to Kostadinka, Karin and Dagmar for the administrative help.

I am grateful to present and past members of the Sommer lab. Particularly, I thank Christian, Amit, Praveen, Gabriel and Neel for bioinformatics support and Cameron, Vladislav, Olga, Xiaoyue, Melanie, Matthias, Arpita, Igor, Lisa, Isabella, Eduardo, Andreas, Akira, James and Robbie for their expertise.

Special thanks goes to my friends outside of the lab, Dr. Anna Hovhanyan, Dr. Zhasmine Mirzoyan, Artur Ghazaryan and Dr. Vladislav Grishkevich. Their support during my PhD scientifically and morally was always pushing me forward. Finally, I am grateful to my family for their patience and support throughout my studies.

This work was granted by Max Planck Society.

Curriculum Viate

Name: Vahan Serobyan

Education

11/2009-	Max Planck Institute for Developmental Biology, Department of Integrative Evolutionary Biology Tübingen, Germany
11/2007-10/2009	Ben-Gurion University of Negev, Israel The Jacob Blaustein Institutes for Desert Research, Albert Katz International School for Desert Studies, Department of Agriculture and Biotechnology of Drylands
09/2002-06/2006	Yerevan State University, Armenia Faculty of Biology

Publications

McGaughran A., Rödelsperger C., Grimm D. G., Meyer J.M., Moreno E., Morgan K., Leaver M., **Serobyan V.**, Rakitsch B., Borgwardt K.M., Sommer R.J. (submitted 2015) Genome-wide Profiles of Diversification and Genotype-phenotype Association in Nematodes from La Réunion Island

Praveen B., Rödelsperger C., Prabh N., **Serobyan V**., Markov G., Hirsekorn A., Dieterich C. (submitted 2015) Ancient gene duplications have shaped developmental stage-specific expression in Pristionchus pacificus

Serobyan V., Xiao H., Rödelsperger C., Namdeo S., Röseler W., Witte H. and Sommer R.J. (submitted 2015) Chromatin remodeling and antisense-mediated up-regulation of the developmental switch gene *eud-1* control a predatory feeding decision

Serobyan V., Ragsdale E.J. and Sommer R.J. (2014). Adaptive value of a predatory mouth-form in a dimorphic nematode. *Proceedings of the Royal Society of London B* 281: 20141334

Serobyan V., Ragsdale E.J., Müller M. R. and Sommer R.J. (2013). Feeding plasticity in the nematode *Pristionchus pacificus* is influenced by sex and social context and is linked to developmental speed. Evolution & Development 15: 161-170. (Cover feature)

Khokhlova, I.S., **Serobyan, V.**, Degen, A.A and Krasnov, B.R. (2011). Discrimination of host sex by a haematophagous ectoparasite. *Animal Behaviour* 81: 275-281.

Khokhlova, I.S., **Serobyan, V.**, Degen, A.A and Krasnov, B.R. (2010). Host gender and offspring quality in a flea parasitic on a rodent. *Journal of Experimental Biology* 213: 3299-3304

Khokhlova I.S., **Serobyan V.**, Krasnov B.R. and Degen A.A. (2009). Effect of host gender on blood digestion in fleas: mediating role of environment. *Parasitology Research* 105: 1667-1673

Khokhlova I.S., **Serobyan V.**, Krasnov B. and Degen A.A. (2009). Is the feeding and reproductive performance of the flea, Xenopsylla ramesis, affected by the gender of its rodent host, Meriones crassus? *Journal of Experimental Biology* 212: 1429-1435

Conferences

Poster: **Serobyan V.** and Sommer R. J. “Epigenetic regulation of a developmental switch gene” EMBO Conference Series: “Chromatin and epigenetics” Heidelberg, Germany (6-10 May 2015)

Poster: **Serobyan, V.**, Ragsdale, E. J., and Sommer, R. J. “Phenotypic plasticity and predatory feeding: ecology and evolution of nematode killing.” Gordon Research Conference: “Predator-prey interactions: from genes to ecosystems to human mental health.” Ventura, CA, USA (6-10 January 2014).

Poster: **Serobyan V.**, Ragsdale E. J., Müller M. R., and Sommer R. J. “Sex plays a role in feeding structure plasticity in *Pristionchus pacificus*.” EMBO Conference Series: “Chromatin and epigenetics” Heidelberg, Germany (8-12 May 2013)

Arakelyan M. S., Danielyan F. D. and **Serobyan V**. “Age, growth and longevity of *Eremias strauchi* from Armenia” Theses of the Ukraine’s Herpetological Societies, Kiev: 19-22 (9-12 October, 2005)

Appendix

EVOLUTION & DEVELOPMENT 15:3, 161–170 (2013)
DOI: 10.1111/ede.12030

Feeding plasticity in the nematode *Pristionchus pacificus* is influenced by sex and social context and is linked to developmental speed

Vahan Serobyan, Erik J. Ragsdale, Manuela R. Müller, and Ralf J. Sommer*
Department of Evolutionary Biology, Max Planck Institute for Developmental Biology, Spemannstraße 37, Tübingen, Germany
*Author for correspondence (e-mail: ralf.sommer@tuebingen.mpg.de)

SUMMARY The increasing evidence for a role of developmental plasticity in evolution offers exciting prospects for testing interactions between ecological and developmental genetic processes. Recent advances with the model organism *Pristionchus pacificus* have provided inroads to a mechanistic understanding of a developmental plasticity. The developmental plasticity of *P. pacificus* comprises two discontinuous adult mouth-forms, a stenostomatous ("narrow mouthed") and a eurystomatous ("wide mouthed") form, the latter of which is structurally more complex and associated with predatory feeding. Both forms are consistently present in populations, but fundamental properties guiding fluctuations in their appearance have been poorly understood. Here, we provide a systematic characterization of the mouth plasticity in *P. pacificus*, quantifying a strong sexual dimorphism and revealing that, in an inbred genetic background, maternal phenotype is linked to that of male offspring. Furthermore, cues from conspecifics influenced the developmental decision in juvenile nematodes. Separating individuals from a population resulted in a lower eurystomatous frequency, which decreased incrementally with earlier isolation. Finally, the time to the reproductively mature stage was, in the presence of an abundant bacterial food supply, less for stenostomatous than for eurystomatous individuals, suggesting the potential for a fitness trade-off between developmental time and breadth of diet. This study provides a baseline understanding of the mouth dimorphism in *P. pacificus* as a necessary reference point for comparative analysis.

INTRODUCTION

The ability of a single genotype to exhibit major phenotypic differences is becoming increasingly recognized as a driver of novelty and the diversity of form (West-Eberhard 2003). The link between polyphenism and evolution is supported by numerous case studies, as highlighted in several recent reviews (Fusco and Minelli 2010; Moczek 2010; Pfennig et al. 2010; Moczek et al. 2011). It has been argued that developmental plasticity facilitates morphological innovations that result in new traits and, simultaneously, allow for novel interactions in the environment (West-Eberhard 2003). Beyond this theoretical framework developed for understanding the role of developmental plasticity in the origin of new traits, experimental evidence has revealed some specific genetic mechanisms that are involved in the accommodation of polyphenic traits (Suzuki and Nijhout 2006) or are associated with their expression (Braendle et al. 2005; Snell-Rood and Moczek 2012).

In the nematode *Pristionchus pacificus*, plasticity of feeding structures was coupled with known developmental pathways (Bento et al. 2010). The polyphenism of *P. pacificus*, as in other species of the family Diplogastridae, consists of a stenostomatous ("narrow mouthed") and a eurystomatous ("wide mouthed") form, which differ in the number and shape of teeth and in the complexity of other mouth armature (Fig. 1). The dimorphism is thought to relate to feeding differences, whereby the eurystomatous form is associated with predation of other nematodes (Kiontke and Fitch 2010). Besides its ecological significance, the genetic control of the mouth dimorphism has begun to be investigated. Specifically, it was shown that the incidence of the stenostomatous form in *P. pacificus* was higher in populations treated with Δ7-dafachronic acid (DA), a steroid hormone that inhibits the formation of a resistant, alternative juvenile ("dauer") stage by acting on the nuclear hormone receptor DAF-12 (Bento et al. 2010). Correspondingly, starvation conditions or the application of pheromone derived from high-density cultures induce both the eurystomatous form and dauer formation (Bento et al. 2010). However, mechanisms for the mouth and life-stage dimorphisms do not completely overlap, as the dauer-promoting transcription factor DAF-16/FOXO has no effect on the mouth phenotype (Ogawa et al. 2011). The unraveling of signaling pathways directly influenced by environmental parameters thus allows exciting new tests of the interaction between developmental and

© 2013 Wiley Periodicals, Inc.

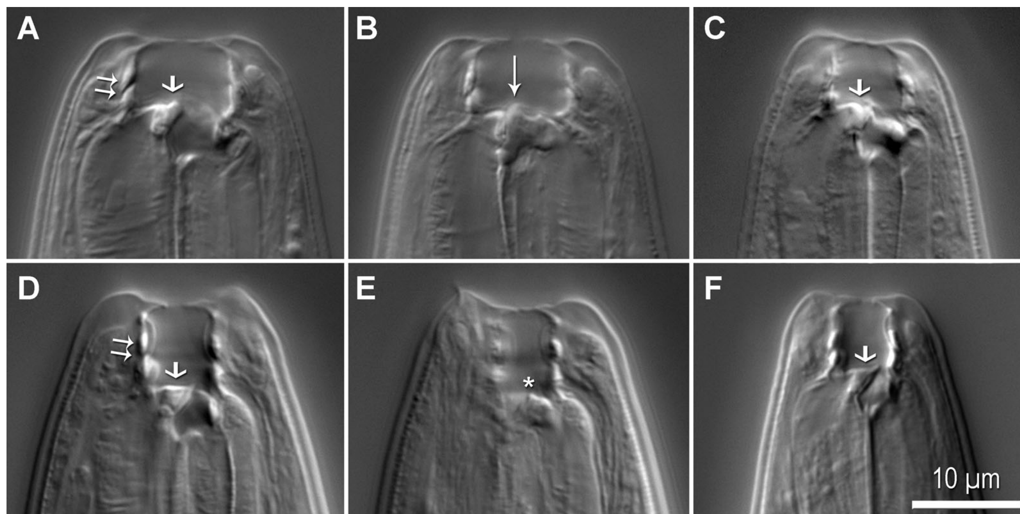


Fig. 1. DIC micrographs of the dimorphic stoma (mouth) of *Pristionchus pacificus*. All images are at same scale. Dorsal is left in all images. (A and B) Eurystomatous hermaphrodite in the sagittal and right sublateral planes, respectively. (C) Eurystomatous male in the sagittal plane. (D and E) Stenostomatous hermaphrodite in the sagittal and right sublateral planes, respectively. (F) Stenostomatous male in the sagittal plane. The eurystomatous and stenostomatous forms differ in the width of the mouth but also in several discrete characters. Short arrows indicate dorsal tooth, which is claw-like in the eurystomatous form (A–C) and thin and symmetrical, or flint-like, in the stenostomatous (D–F). Long arrow indicates opposing, claw-like subventral tooth, which is absent (asterisk) in the stenostomatous form. Stomatal walls (double arrows) are rigid and more highly sclerotized in the eurystomatous form, in contrast to their beaded appearance in the stenostomatous form. Notwithstanding a size difference in the mouth by sex, mouth-forms are qualitatively identical in the two sexes, as highlighted by the shape of the dorsal tooth in the two male forms (C and F).

ecological processes (Schlichting and Pigliucci 1998; Sommer and Ogawa 2011).

Among models for polyphenism, *P. pacificus* has a powerful set of analytical tools available to it. As a well-established satellite model to that of *Caenorhabditis elegans*, *P. pacificus* enables comparative developmental and genetic studies (Sommer 2009). Genetic analysis of dimorphism in *P. pacificus* is made feasible by androdioecious reproduction (Sommer et al. 1996), genetic and physical maps of the genome (Srinivasan et al. 2002, 2003), a sequenced and annotated genome (Dieterich et al. 2008), and the capability for forward genetics (Zheng et al. 2005; Schlager et al. 2006) and DNA-mediated transformation (Schlager et al. 2009). The recent identification of another developmental regulator, the cyclic-GMP-dependent protein kinase *egl-4*, with a mutant mouth-dimorphism phenotype has further demonstrated this genetic tractability (Kroetz et al. 2012).

Besides its amenability to genetics studies, *P. pacificus* derives power as an animal model for developmental plasticity from being rigorously quantifiable. The short generation time and large brood size of *P. pacificus* make it amenable to high-throughput screens. The phenotype can, therefore, be studied as the frequencies of forms in a population that change in statistically testable ways. Quantitative analysis of phenotypic plasticity is of significance for an ultimate understanding of the interplay of the environment and intrinsic genetic and molecular mechanisms.

Despite the inroads this system gives to understanding the precise genetic basis and evolutionary consequences of a dimorphism, the factors that guide the fluctuations in the trait are still poorly understood. The frequencies of the two forms are apparently stochastic in populations, even under consistent food and ambient conditions (Bento et al. 2010). Although starvation, dauer pheromone, or Δ7-DA can perturb these frequencies, both forms normally occur in every generation (Bento et al. 2010). What other genetic or environmental factors might influence the development of the two forms are still unknown. For example, an open question is that of sexual dimorphism of the mouth-form plasticity in *P. pacificus*. The reported absence of eurystomatous males in some other diplogastrid genera (von Lieven and Sudhaus 2000) has suggested this possibility. Hints of possible cross-generational effects and the precise influence of density-specific cues (Bento et al. 2010) are also unresolved. Here we have endeavored to thoroughly characterize the mouth-form plasticity of *P. pacificus* and thereby provide the necessary foundation for budding research on this system.

MATERIALS AND METHODS

To provide a rationale for standardization in further studies on the *P. pacificus* mouth dimorphism, we have established a method for accurately characterizing the dimorphism phenotype under a defined set of conditions.

Pristionchus pacificus

All experiments were conducted with the inbred, wild-type reference strain of *P. pacificus*, RS2333 (=PS312). Post-embryonic development of *P. pacificus* consists of four juvenile stages (J1-J4), with the first molt (J1 to J2) occurring within the egg (von Lieven 2005). Sex determination in *P. pacificus* is by an XX:X0 system, in which males occur spontaneously as a result of accidental X-chromosome non-disjunction (Sommer et al. 1996). The appearance of spontaneous males can then lead to the spread of males throughout a population by sexual reproduction. In standard laboratory culture the frequency of spontaneous males in strain RS2333 is about 0.5% (Click et al. 2009).

Nematodes were maintained on nematode growth medium agar plates seeded with a lawn grown from 400 μl (or 100 μl for crossing plates) of *Escherichia coli* strain OP50 in L-Broth. All plates were kept at 20°C. Plates showing any signs of bacterial or fungal contamination were excluded from experiments. To prevent any mechanical stresses during handling of nematodes, juveniles were picked with a buffer of viscous bacterial solution derived from OP50 lawns, such that direct physical contact with nematodes was reduced or eliminated. To avoid possible trans-generational effects of starvation or other environmental aberrations, nematodes were cultured under well-fed, non-crowded conditions for at least three generations before picking nematodes for cultures referred to as “source plates” herein. Source plates were each established from five J4 (virgin) hermaphrodite progenitors; nematodes of the ensuing generation were used to start all experiments. Thus all nematodes went through at least four generations in healthy culture, the most recent generation encountering a roughly standard population density (i.e., the progeny of five hermaphrodites), prior to experiments.

Phenotype scoring

The mouth dimorphism of *Pristionchus* spp. is discontinuous and is manifest and developmentally irreversible at the adult stage (Hirschmann 1951). Phenotypes were scored according to morphological differences detailed by von Lieven and Sudhaus (2000) and Kanzaki et al. (2012). Differences were sufficient to positively identify either of the two forms, such that neither form was scored by default. Characters used to discriminate between eurystomatous and stenostomatous individuals, respectively, were (Fig. 1): (i) the presence versus absence of a subventral tooth; (ii) a claw-like versus flint-like (i.e., dorsoventrally symmetrical) dorsal tooth; (iii) strongly versus weakly sclerotized stomatal walls; and (iv) a wide versus narrow stoma (mouth). The discrete, non-overlapping characters (i) and (ii) are sufficient to distinguish the two forms in *P. pacificus* as well as in all other examined *Pristionchus* species (E. J. R., pers. obs.). Intermediate states are possible in characters (iii) and (iv), although the polar ends of these character distributions are always correlated with the respective states for characters (i) and (ii). True intermediates, namely within or between characters (i) and (ii), are apparently rare ($<0.1\%$ of specimens examined; E. J. R., pers. obs.); they were not found in the present study and thus not included in counts. Phenotypes were authoritatively determined by differential interference contrast (DIC) microscopy on a Zeiss Axioskop. To enable higher throughput in screens, phenotypes were also scored using Zeiss Discovery V.12 and V.20 stereomicroscopes and then supplemented where necessary with DIC microscopy.

Phenotype characterization by sex, parentage, and maternal phenotype

The mouth-form phenotype of *P. pacificus* was characterized by the following measurements: (i) eurystomatous frequency of spontaneous males; (ii) eurystomatous frequency of hermaphrodites of the same cohorts as spontaneous males; (iii) eurystomatous frequency of male progeny from crosses; (iv) eurystomatous frequency of hermaphroditic progeny from crosses (“cross-hermaphrodites”); and (v) eurystomatous frequency of hermaphroditic progeny from selfing mothers (“self-hermaphrodites”).

Taking these measurements in a controlled genetic and environmental background followed the occurrence of spontaneous males, due to the rarity of these males in laboratory culture. To begin, source plates, each containing cohorts born of five J4 hermaphrodites, were screened for spontaneous males after 6 days of growth. After successfully collecting and screening several ($n = 40$) spontaneous males, which were never crossed but are included in the analyzed samples, the following experimental screen was conducted for all subsequently isolated spontaneous males. The final sample of spontaneous males ($n = 125$) was obtained after screening 260 source plates. Each spontaneous male found was transferred to a crossing plate, where it was paired with a J4 (virgin) hermaphrodite randomly picked from the same source plate and then let to mate overnight. In parallel, five additional J4 (virgin) hermaphrodites were randomly picked from the same source plate onto their own individual plates. On the following day, males were recovered and screened for their mouth-form phenotype. Both the crossed hermaphrodites and the five virgin hermaphrodites picked from the same source plate were retained on culture plates overnight to lay eggs. Two days following the initial cross, crossing and virgin hermaphrodites were recovered and screened for their mouth form. Six days after crossing, mouth-form phenotypes were screened for cross-broods, which included hermaphrodites and males. Additionally, the mouth forms were screened in a self-brood of one mother of the same cohort (i.e., one of the five hermaphrodites isolated in parallel) and whose mouth-form was the same as the hermaphrodite in the cross; if such a mother was not found, then a corresponding self-brood was not included. In this manner, self- and cross-progeny of mothers of the same phenotype and source population could be directly compared.

Table 1. The eurystomatous frequency of *Pristionchus pacificus* under a laboratory culturing regime and characterized by sex, parentage, and maternal phenotype

		Self-hermaphrodites		Cross-hermaphrodites		Cross-males	
		Mean ± SE	*n* (*N*)	Mean ± SE	*n* (*N*)	Mean ± SE	*n* (*N*)
	Total	86.33 ± 2.55	19 (1352)	83.24 ± 3.06	28 (1482)	21.28 ± 3.87	28 (580)
Maternal phenotype	Eu	87.61 ± 2.13	15 (917)	85.86 ± 2.57	18 (1015)	29.65 ± 3.53	18 (416)
	St	83.15 ± 7.50	4 (435)	76.70 ± 8.53	10 (467)	0.35 ± 0.35	10 (164)

Values correspond to results in Fig. 2B. Sample size (*n*) of plates and total number (*N*) of individuals screened are given. Eu, eurystomatous; St, stenostomatous.

Entire broods resulting from 2 days of oviposition were screened and needed to comprise at least 50 individuals to be included in the experiment. To be considered a "successful" cross and thus included in the experiment, broods must have been at least 20% males. Sample sizes for all categories of individuals are given in Table 1. Morphological mutant lines were not used to distinguish hermaphroditic self- from cross-progeny in cross plates to avoid biases that could be introduced by pleiotropic effects on the mouth phenotype in those mutants (Müller and Sommer, unpublished data).

Because of the difficulty in distinguishing cross- from self-hermaphrodites, we additionally tested for differences between cross- and self-progeny by crossing males carrying a stably transmitted reporter gene to mother hermaphrodites. The reporter used was *Ppa-egl-20*::rfp (strain RS2597; Kienle and Sommer 2013), which is expressed in the tail at all life stages (Schlager et al. 2009) and which was confirmed to be transmitted with 100% penetrance ($n = 373$). Prior to experiments, reporter populations were cultured for at least four generations under a consistent population density as described above. To test the effect of paternity on the mouth-form, crosses were established between one *Ppa-egl-20*::rfp male and one young adult hermaphrodite of the reference strain. Fluorescently reporting F1 hermaphrodites were identified as cross-progeny, whereas all non-reporting hermaphrodites were considered self-progeny. As a control for the neutrality of the reporter gene toward the mouth-form phenotype, we also screened the self-progeny of each mouth-form that were produced by one young adult hermaphrodite per mating plate. Sample sizes were 19 and 15 replicates (plates) for crosses with eurystomatous and stenostomatous mothers, respectively, and were 12 and 11 for *Ppa-egl-20*::rfp selfing plates with eurystomatous and stenostomatous mothers, respectively.

Effect of population cues on the adult phenotype decision

To obtain juveniles for testing the effect of isolation on the mouth-form plasticity, five source plates were allowed to grow for 7 days (1.5 generations), such that juveniles of all stages were available in a single population. From each of these plates, 10 individuals of each juvenile stage (J2, J3, and J4) were transferred to new individual plates. After completing development in isolation, individuals were screened for their mouth form. As a control, 10 randomly picked young but already matured hermaphrodites from each of the same source plates were screened for their mouth form. The experiment was performed in triplicate to result in a sample of 150 individuals isolated per life-stage except J2, for which the sample size was 144 hermaphrodites after excluding failed developers and spontaneous males.

Developmental timing of mouth forms

To collect and synchronize juveniles for timing of their development, eggs were transferred from multiple source plates to a single new plate. J2 individuals that hatched on this plate within 2 h were transferred to their own individual plates and screened for their developmental stage once a day. J2 hatchlings were picked from the same batch of eggs at three different starting times, which were separated by 4-h intervals, to make a total of 150 individuals. After the first individuals reached the J4 stage, all animals were screened every 4 h until becoming adults, after which they were screened for their mouth form. Duration of development was calculated as the time from hatching to the adult stage. Those animals that did not molt to the J3 stage within 72 h were presumed to not have recovered from handling and were excluded from the experiment. Because of the fragility of young hatchlings, several were unable to complete the experiment: after premature deaths, failed developers, extremely late developers (see below), and one spontaneous male, the total number of samples was 141 ($n = 68$ eurystomatous, $n = 73$ stenostomatous).

Statistical analyses

Count data were obtained in two experiments: (i) phenotypes of hermaphrodites and spontaneous males individually picked for crossing experiments and (ii) isolation of individuals at different life-stages. Differences in the proportion of eurystomatous individuals from these experiments were tested using Fisher's exact test. Confidence intervals for all count data were estimated by a binomial test.

In all other experiments characterizing the mouth-form phenotype, each sample was an entire plate for which the eurystomatous frequency was recorded. Prior to statistical tests, an arcsine transformation was applied to proportional variables. Distributions of these variables after arcsine transformation did not deviate from normality (Kolmogorov-Smirnov test, $P > 0.1$ for all). To test whether (i) maternal mouth-form, (ii) cross type (self vs. cross), or (iii) sex of offspring had an effect on the mouth-form decision of offspring, we performed three-way ANOVA where these three variables were independent. Additionally, one-way ANOVA was used to separately determine whether maternal mouth-form influenced the phenotype of (i) self-hermaphrodite, (ii) cross-hermaphrodite, or (iii) male offspring. Differences in the proportions of eurystomatous animals were tested using one-way ANOVA with maternal mouth-form as the independent variable.

In a separate experiment, where differences between self- and cross-progeny were tested by crossing *Ppa-egl-20*::rfp males to wild-type hermaphrodites, one-way ANOVA was used to individually test for effects of (i) maternal mouth-form, (ii) cross type (self vs. cross), and (iii) the *Ppa-egl-20*::rfp transgene on hermaphroditic self-progeny.

In the analysis of developmental timing results, distributions of groups (times for eurystomatous vs. for stenostomatous) initially deviated from normality (Kolmogorov-Smirnov test, $P < 0.01$). Inspection for outliers identified three extremely late developers (developmental times of 88, 88, and 92 h) that matured in a second wave later than all others of both forms (non-outlier maximum = 72 h) and became stenostomatous. Extremely late developers may have been due to stress caused by trauma during handling, suggested by their comparatively small adult body size. Whether the stenostomatous program was a cause, result, or coincidence of an abnormal development rate in those individuals is unclear. After removing extreme cases, the two distributions of the developmental times no longer deviated from normality (Kolmogorov-Smirnov test, $P > 0.05$). Therefore, Student's *t*-test was used to compare the mean maturation time for independent samples (eurystomatous vs. stenostomatous).

Count data were analyzed with R; all other statistical tests were implemented in the program Statistica v. 9 (Statsoft). All figures present untransformed data. For data that were transformed for statistical analysis, whiskers represent the standard error estimated for untransformed data. All percentages given in the text are the frequency of eurystomatous nematodes. Other statistics are given in Table 1.

RESULTS

Dimorphism differs by sex and maternal phenotype

With our experimental design we sought to simultaneously test for: (i) the presence and extent of sexual dimorphism in the frequencies of the two forms; (ii) any correlation between the phenotypes of parents (i.e., mothers) and offspring; and (iii) any differences between offspring of selfing hermaphrodites and those from crosses with males. The dimorphism phenotype of *P. pacificus* was characterized for clones from the same culture conditions and, for all screens downstream of the isolation of J4 hermaphrodite and spontaneous male parents, the same parentage.

Addressing our first question, sexual dimorphism in mouth-form frequencies was evident in comparisons under all conditions. A clear difference was found for individual nematodes isolated from the same culture populations, where hermaphrodites were 71.6% ($n = 431$) and spontaneous males 11.2% ($n = 125$) eurystomatous (Fisher's exact test, $P < 10^{-6}$; Fig. 2A). Three-way ANOVA of the mean eurystomatous frequency of broods, which consisted of males and hermaphrodites under the same environmental conditions and of the same known parentage, identified a phenotypic difference between the sexes in the offspring ($F_{1,73} = 10.2$, $P < 0.0005$; a vs. bc, Fig. 2B).

An unexpected maternal effect resulted in an additional difference in the plasticity in offspring ($F_{1,73} = 5.12, P < 0.05$). Males from eurystomatous mothers showed a significantly ($F_{1,18} = 17.32$, $P < 0.001$) higher eurystomatous frequency (29.7%) than males from stenostomatous mothers (0.3%; b vs. c, Fig. 2B). No difference between hermaphrodites born of the two

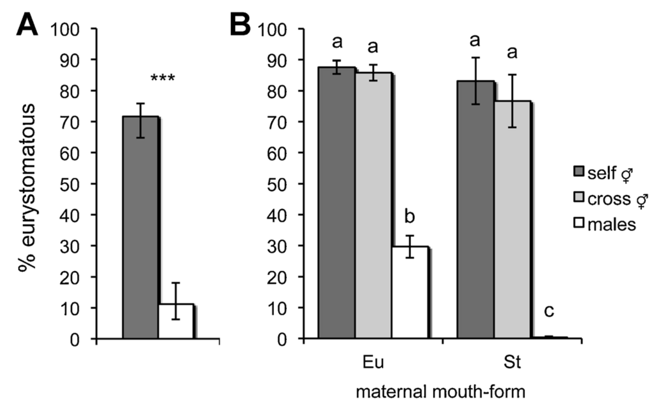


Fig. 2. The mouth-form phenotype of *P. pacificus* by sex, parentage, and maternal phenotype. (A) The total eurystomatous frequencies of spontaneous males (open bars) that is males produced by X-chromosome non-disjunction, and of hermaphrodites (dark gray) occurring in the same culture populations as spontaneous males. Difference is significant by Fisher's exact test ($^{***}P < 10^{-6}$). Whiskers represent a 95% confidence interval. (B) The mean eurystomatous frequencies of self-hermaphrodites (dark gray), cross-hermaphrodites (light gray), and cross-males (open). Cross-progeny are from spontaneous males and co-occurring hermaphrodites; self-progeny is from virgin co-occurring hermaphrodites. Each type of offspring is additionally distinguished by maternal phenotype being eurystomatous (Eu) or stenostomatous (St). Significant differences were detected by three-way ANOVA (a vs. bc, $P < 0.0005$) and one-way ANOVA (b vs. c, $P < 0.001$). Whiskers represent the standard error.

maternal forms was statistically supported ($F_{1,47} = 1.23$, $P > 0.05$), indicating that the effect in male offspring drove the difference found in the general comparison. Because hermaphrodites were crossed to clonal (spontaneous) males, and given the inability to artificially select for either mouth-form in RS2333 (PS312) by self-reproduction (Bento et al. 2010), genetic variation in this highly inbred strain is considered to be low. Therefore, such a correlation of phenotypes between mothers and sons cannot be attributed purely to genetic inheritance. Unfortunately, we were unable to test for an effect of paternal phenotype due to the inadequate number of eurystomatous males available in culture.

Finally, the phenotype of self-hermaphrodites did not differ from that of cross-hermaphrodites from crosses to spontaneous males ($F_{1,73} = 0.67$, $P > 0.05$). However, a real difference could have been underestimated by the inaccuracy built into the reproductive mode of *P. pacificus*: because hermaphroditic offspring of crossing mothers may also include self-progeny, any difference present would be partially hidden by the inclusion of unidentifiable self-offspring in counts of cross-offspring. Therefore, we performed crosses using a marker, *Ppa-egl-20*::rfp, which definitively distinguished self- from cross-progeny and which was confirmed to be neutral with respect to the mouth-form frequency ($F_{1,55} = 1.52$, $P > 0.2$; Fig. 3). This test confirmed that there was no difference in the mouth-form phenotype between self- and cross-progeny ($F_{2,31} = 0.21$, $P > 0.2$) nor any correlation of phenotype between mothers and their hermaphroditic self-progeny ($F_{1,32} = 0.22$, $P > 0.2$) or cross-progeny ($F_{1,32} = 0.09$, $P > 0.2$; Fig. 3). A similar comparison could also not be made for males, as identifying the maternal phenotype of self-cross (i.e., spontaneous) males was not feasible.

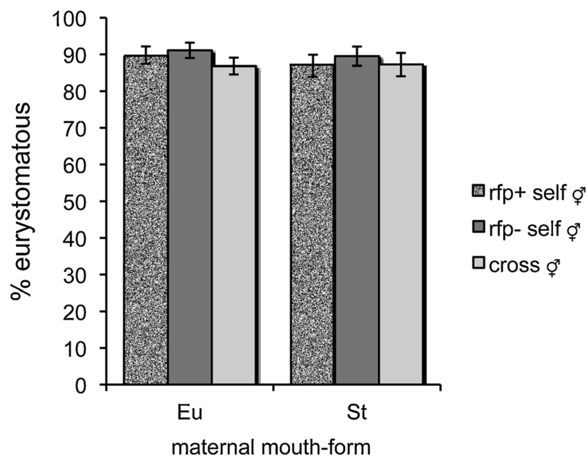


Fig. 3. The effect of paternity on hermaphroditic progeny as tested by crosses with a *Ppa-egl-20*::rfp reporter. The mean eurystomatous frequencies of *Ppa-egl-20*::rfp self-hermaphrodites (textured), wild-type self-hermaphrodites (dark gray), and *Ppa-egl-20*::rfp cross-hermaphrodites (light gray) are shown. Cross-progeny are from *Ppa-egl-20*::rfp males and wild-type hermaphrodites. Each type of offspring is additionally distinguished by maternal phenotype being eurystomatous (Eu) or stenostomatous (St). No differences by (i) maternal mouth-form, (ii) cross type (self vs. cross), and (iii) the *Ppa-egl-20*::rfp transgene on hermaphroditic self-progeny were detected by one-way ANOVA ($P > 0.2$ for all). Whiskers represent the standard error.

Isolation from conspecifics influences the developmental decision

Characterizing the mouth phenotype by sex revealed a putative discrepancy between hermaphrodites individually picked from populations with spontaneous males (71.7% eurystomatous) and their hermaphroditic self-progeny (83.2% from stenostomatous and 87.6% from eurystomatous mothers; Fig. 2B). Given the otherwise standardized genetic and environmental conditions, only one consistent difference between the two experiments was obvious: that hermaphrodites picked together with spontaneous males were always isolated as J4 juveniles, to ensure their virginity, whereas those in broods had always matured to the adult stage in a social context. Because pheromone levels are known to increase the eurystomatous frequency in culture (Bento et al. 2010), we suspected that isolation as J4 from cues given by conspecifics may have resulted in a lower likelihood of becoming eurystomatous. Therefore, we next tested whether exposure through different life-stages to signals of population density would reveal differences in sensitivity for the decision of the adult phenotype.

Isolation of each post-eclosion juvenile stage from multiple, synchronized populations of similar densities led to different phenotypes in the adult (Fig. 4). Nematodes isolated as adults,

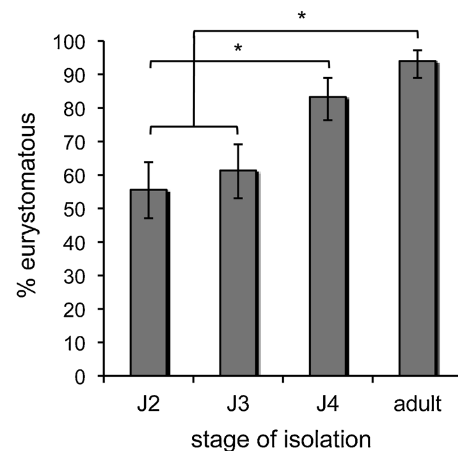


Fig. 4. The mouth-form phenotype of *P. pacificus* hermaphrodites when isolated from populations at different life-stages. Individuals were transferred at one of the three post-eclosion juvenile stages (J2–J4) or allowed to reach the adult stage together with conspecifics. Total eurystomatous frequencies are shown. Significant differences ($^{*}P < 0.05$) are according to Fisher's exact test. Whiskers represent a 95% confidence interval.

after all chances to alter the phenotype decision had passed, showed the highest eurystomatous frequency (93.3%) of any isolated stage. In contrast, those isolated as J2 or J3 juveniles showed significantly lower eurystomatous frequencies (57.3% and 61.7%, respectively) than those isolated as adults (Fisher's exact test, $P < 0.05$). Nematodes isolated during J4 showed a eurystomatous frequency (83.3%) intermediate between those isolated as J3 and as adults and which was different from that of isolated J2 (Fisher's exact test, $P < 0.05$). There was no significant difference between juveniles isolated as J2 and those as J3. Thus, sensitivity to external cues decreased gradually with successive juvenile stages and persisted at least as late as the J3 stage.

Developmental timing of the two forms

The duration of an inherent developmental program could govern the amount of exposure to external cues, and so any difference between the two forms could influence the interaction among developing nematodes. To isolate the effect of post-embryonic developmental time, we tested for differences in the absence of population cues. Tracking the developmental time of nematodes isolated as J2 hatchlings ($\leq$2 h old) revealed that individuals that became stenostomatous developed significantly more rapidly ($T_{139} = -5.67$, $P < 0.05$) than those that became eurystomatous (Fig. 5): the mean ($\pm$ SD) time to adulthood was 55 ± 3 h in stenostomatous as compared to 61 ± 2 h in eurystomatous nematodes. The time from completion of the J3–J4 molt to that of the final molt, a period of 7 ± 5 h in stenostomatous and 12 ± 5 h in eurystomatous nematodes, can account for most of this difference. Thus, the two adult phenotypes were clearly correlated with different rates of post-embryonic development, particularly at the last juvenile stage and final molt.

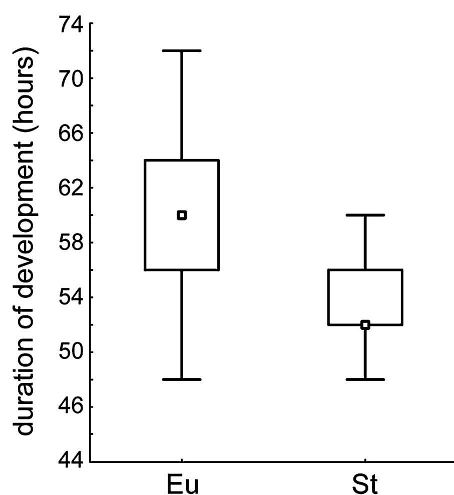


Fig. 5. The duration of post-embryonic development in the two mouth forms of *P. pacificus* at 20°C. Individuals were isolated as hatchlings ($\leq$2 h old) and their development tracked every 4 h until reaching the adult stage. Upon becoming adults, their mouth-form phenotype was recorded. Box plots show the median (center square), the lower and upper quartiles (box bounds), and the non-outlier range limits (whiskers) of the period from hatching to maturity. The difference between the eurystomatous (Eu) and stenostomatous (St) forms in duration of developmental time is significant (Student's *t*-test, $P < 0.05$).

DISCUSSION

The mouth dimorphism of *P. pacificus* is governed by a complex of sexual parameters and external cues. Although some mechanistic developmental context has been given to the phenomenon (Bento et al. 2010), the plasticity in general was not previously well described. In the present study, some of the variables governing the seemingly stochastic occurrence of its two forms were identified. A basic understanding of the dimorphism trait will be indispensible for future work on this system.

Sex plays a role in the feeding-structure plasticity

Although sexual dimorphism in the mouth plasticity in *P. pacificus* was hinted by an apparent lack of eurystomatous males in some diplogastrid taxa (von Lieven and Sudhaus 2000), the work herein is the first to systematically test and quantify such a difference. Not only is recognizing a precise difference between the sexes necessary for a complete understanding of the trait, it may narrow the search for mechanisms by warranting attention to sex-linked developmental processes. Herein we report a strong difference between hermaphrodites and males, which in populations are dominated by the eurystomatous and stenostomatous forms, respectively. It is likely that the rarity of outcrossing events shown in the laboratory (Click et al. 2009) and inferred in the wild (Morgan et al. 2012) for *P. pacificus* undermines the selection potential conferred by male-mediated differences within this species. However, sex-related effects could play a much larger role in the ecological divergence of other *Pristionchus* species, most of which are gonochoristic (Mayer et al. 2007; Kanzaki et al. 2012). Consequently, any such role would also be predicted for the evolution of hermaphroditic *Pristionchus* species from gonochoristic ancestors.

The predominance of the eurystomatous form among *P. pacificus* hermaphrodites was a surprising contrast to the findings of Bento et al. (2010), who reported hermaphrodites as being mostly stenostomatous (approximately 30% Eu) in their control experiments. Given the results obtained in this study, this discrepancy might be explained in several ways. First, the culturing regimen and thus possible cross-generational effects were controlled differently between studies. Second, a likely cause of the discrepancy is observer differences, which can never

be completely ruled out. The method used by Bento et al. to discriminate phenotypes emphasized head shape and stoma width, although these features can be variable as compared to the qualitative differences of the teeth (von Lieven and Sudhaus 2000; Fig. 1). Finally, it should be mentioned that another possibility is that of mutation accumulation in the strains used in the different studies. For the present study and in ongoing work with *P. pacificus*, strains used in experiments are freshly thawed from a frozen voucher once per year in order to minimize mutation accumulation that might affect plastic traits that are under strong environmental influence. Additionally, the number of animals used in the experimental set-up should be rigorously controlled, as the density experiments described above (Fig. 4) and the higher eurystomatous frequency induced by increased pheromone concentrations (Bose et al. 2012) both suggest that the number of progeny could influence mouth-form ratio. Taken together, we recommend the protocol used in this study as a general guideline for future studies to control for culture history and population density of source plates, mating status of mothers, the number of mothers used to start an experiment, and the stage of isolated nematodes.

A correlation between the phenotypes of mothers and sons in a genetically identical background is an intriguing result to explain. In an early study of the *Pristionchus* dimorphism, Hirschmann (1951) set up various crosses by parental mouth-form to observe, among other variables, the mouth forms of the offspring. However, because of the irregular complexity of the sampling and experimental scheme in that study, we could not interpret a similar correlation from her results. It is possible that paternal phenotype also has an additional influence on the offspring phenotype, although the scarceness of eurystomatous males prevented our testing this idea. The correlation we observed between mothers and sons could be due to hormonal cues encountered in utero or perhaps some signaling input inherited through the germline. The operation of cross-generational epigenetic effects (Greer et al. 2011; Johnson and Spence 2011; Rechavi et al. 2011; Shirayama et al. 2012) in specifying dimorphism phenotypes is an interesting possibility to test.

Conspecific cues post-embryonically influence adult morphology

Separating individuals of *P. pacificus* from their siblings showed that the presence of a population influences the developmental switch within a single generation. This is consistent with findings that "pheromone" purified from dauer-conditioned medium can influence the decision (Bento et al. 2010), but it reveals the activity of cues even when nematodes are well fed and in the absence of stress-induced dauers. Besides pheromonal cues, the introduction of mechanical cues by handling nematodes was also possible. Earlier juvenile stages are more susceptible to trauma, and so this could hypothetically translate to an influence on the developmental decision. However, the normal development to adulthood of almost all individuals, the stenostomatous of which generally develop even faster (Fig. 5), makes this effect unlikely. Furthermore, isolation of different stages showed that the decision could be altered at least as late as the J3 stage. The continuous response indicates that external information can be decreasingly incorporated into developmental regulation networks until the final morphology is executed, as known for cell-fate plasticity in nematode vulva development (Sternberg 2005).

Feeding plasticity differences in an ecological context

Variability in a feeding dimorphism has direct consequences for exploiting an ecological niche. *Pristionchus* species lead a necromenic lifestyle: they are found on beetles and other insects, and upon the death of the carrier they resume development from the dispersal (dauer) stage to proliferate on the host carcass (Herrmann et al. 2006a, b, 2007; Rae et al. 2008; D'Anna and Sommer 2011). This rapidly changing environment should in principle elicit benefits of one form over the other at different stages of change. Natural food sources include numerous types of bacteria (Rae et al. 2008; Weller et al. 2010) and presumably also fungi and other nematode colonizers (Yeates et al. 1993). If the eurystomatous form is, as assumed, a better predator than the stenostomatous form, a density-dependent switch to this form could represent a resource polyphenism in response to signals of increased competition for dwindling microbial resources (Kiontke and Fitch 2010). In this case, an opportunistic switch to a predatory form would enable predation of nematode competitors, possibly including conspecifics, as observed in anuran tadpoles (Pfennig 1990). Given form-specific feeding differences, the sexual differences in the mouth dimorphism in a population could affect the partitioning of resources among conspecifics, possibly leading to an ecological selection for the sexual dimorphism (Shine 1989). Assuming heritability of relevant loci in wild populations, any selection differentials in the dimorphism trait would, therefore, be predicted by theory to result in population divergence under the appropriate selection regime (West-Eberhard 2003). Further work to determine precise feeding differences between the two forms will be crucial for testing functional and evolutionary consequences of the dimorphism in a real ecological setting.

A developmental trade-off?

When given an abundant bacterial food supply, stenostomatous individuals of *P. pacificus* reached the stage of reproductive maturity in less time than eurystomatous individuals. This is the first evidence for a competitive advantage of the stenostomatous form. Because the eurystomatous form can access all known food sources as the stenostomatous form, and presumably more, benefits to retaining the stenostomatous form in evolution were

previously not obvious. A higher feeding efficiency of the stenostomatous form under some conditions could be supposed, although this remains to be tested. Although the stenostomatous form is less complex in its feeding morphology, differential metabolic costs of producing either form can for now only be predicted. However, if present, they could constitute a trade-off in time to maturity versus dietary breadth. Such a trade-off is supported by a difference in the duration of the J4 stage and final molt. Because the final molt is the point at which a discernibly dimorphic morphology is produced, we hypothesize that more time is needed for the organization or secretion of complex eurystomatous mouthparts. Considering the short and otherwise consistently timed life cycle of *P. pacificus*, any real difference in maturation time could theoretically be acted upon by selection. Although both forms grow well on bacteria, it is possible that a difference in developmental time would be exaggerated under more discriminating conditions. Studying the fitness consequences of a particular form on a wider array of food sources and other niche parameters will reveal whether any such trade-offs are plausible and could confer selective advantages.

A model for linking developmental plasticity to micro- and macro-evolution

Establishing a baseline understanding of the mouth dimorphism in *P. pacificus* provides a necessary reference point for comparative analysis. Anchored by a well-characterized reference strain, studies can be expanded into a population genetic context. For example, the collection of hundreds of distinct haplotypes from around the world (Herrmann et al. 2010; Morgan et al. 2012) has enabled a thorough screen for natural variation of the dimorphism, including wild strains highly biased toward either form (Ragsdale, Müller et al., unpublished data). Moreover, the laboratory availability and resolved phylogeny for some 30 new and described species of *Pristionchus* (Mayer et al. 2007, 2009), including a recently discovered cryptic species complex with *P. pacificus* (Kanzaki et al. 2012), will allow macroevolutionary studies of the plasticity. In such a framework, insight gleaned from genetic analyses in one strain of *P. pacificus* could be applied to testing genetic mechanisms at multiple tiers of evolution. An ultimate question to be addressed regard the origin of the novel morphology itself, particularly the teeth that are the hallmark of the eurystomatous form. Whether the discrete forms are the result of canalization from a continuum (Emlen and Nijhout 2000; Nijhout 2003) or the build-up of cryptic genetic variation by "developmental capacitance" (Moczek 2007) is still the subject of speculation, but the advent of *Pristionchus* and Diplogastridae as a model for plasticity and evolution promises exciting opportunities to put theory to the test.

ACKNOWLEDGMENTS

We thank Dr. Cameron Weadick and Vladislav Susoy for helpful criticism and suggestions. We gratefully acknowledge the Alexander von Humboldt Foundation for the support of a fellowship to E. J. R.

REFERENCES

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

Bose, N., et al. 2012. Complex small-molecule architectures regulate phenotypic plasticity in a nematode. *Angew. Chem.* 51: 12438–12443.

Braendle, C., Friebe, I., Caillaud, M. C., and Stern, D. L. 2005. Genetic variation for an aphid wing polyphenism is genetically linked to a naturally occurring wing polymorphism. *Proc. R. Soc. B* 272: 657–664.

Click, A., Savaliya, C. H., Kienle, S., Herrmann, M., and Pires-daSilva, A. 2009. Natural variation of outcrossing in the hermaphroditic nematode *Pristionchus pacificus*. *BMC Evol. Biol.* 9: 75.

D'Anna, I., and Sommer, R. J. 2011. *Pristionchus uniformis*, should I stay or should I go? Recent host range expansion in a European nematode. *Ecol. Evol.* 1: 468–478.

Dieterich, C., et al. 2008. The *Pristionchus pacificus* genome provides a unique perspective on nematode lifestyle and parasitism. *Nat. Genet.* 40: 1193–1198.

Emlen, D. J., and Nijhout, H. F. 2000. The development and evolution of exaggerated morphologies in insects. *Annu. Rev. Entomol.* 45: 661–708.

Fusco, G., and Minelli, A. 2010. Phenotypic plasticity in development and evolution: facts and concepts. *Phil. Trans. R. Soc. B* 365: 547–556.

Greer, E. L., et al. 2011. Transgenerational epigenetic inheritance of longevity in *Caenorhabditis elegans*. *Nature* 479: 365–371.

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

Herrmann, M., Mayer, W. E., and Sommer, R. J. 2006b. Sex, bugs and Haldane's rule: the nematode genus *Pristionchus* in the United States. *Front. Zool.* 3: 14.

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

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

Hirschmann, H. 1951. Über das Vorkommen zweier Mundhöhlentypen bei *Diplogaster lheritieri* Maupas und *Diplogaster biformis* n. sp. und die Entstehung dieser hermaphroditischen Art aus *Diplogaster lheritieri*. *Zool. Jahrb. Abt. Syst.* 80: 132–170.

Johnson, C. L., and Spence, A. M. 2011. Epigenetic licensing of germline gene expression by maternal RNA in *C. elegans*. *Science* 333: 1311–1314.

Kanzaki, N., Ragsdale, E. J., Herrmann, M., Mayer, W. E., and Sommer, R. J. 2012. Description of three *Pristionchus* species (Nematoda: Diplogastridae) from Japan that form a cryptic species complex with the model organism *P. pacificus*. *Zool. Sci.* 29: 403–417.

Kienle, S., and Sommer, R. J. 2013. Cryptic variation in vulva development by *cis*-regulatory evolution of a HAIRY-binding site. *Nat. Commun.* In press. doi: 10.1038/ncomms2711

Kiontke, K., and Fitch, D. H. A. 2010. Phenotypic plasticity: different teeth for different feasts. *Curr. Biol.* 20: R710–R712.

Kroetz, S. M., Srinivasan, J., Yaghoobian, J., Sternberg, P. W., and Hong, R. L. 2012. The cGMP signaling pathway affects feeding behavior in the necromenic nematode *Pristionchus pacificus*. *PLoS ONE* 7: e34464.

Mayer, W. E., Herrmann, M., and Sommer, R. J. 2007. Phylogeny of the nematode genus *Pristionchus* and implications for biodiversity, biogeography and the evolution of hermaphroditism. *BMC Evol. Biol.* 7: 104.

Mayer, W. E., Herrmann, M., and Sommer, R. J. 2009. Molecular phylogeny of beetle associated diplogastrid nematodes suggests host switching rather than nematode-beetle coevolution. *BMC Evol. Biol.* 9: 212.

Moczek, A. P. 2007. Developmental capacitance, genetic accommodation, and adaptive evolution. *Evol. Dev.* 9: 299–305.

Moczek, A. P. 2010. Phenotypic plasticity and diversity in insects. *Phil. Trans. R. Soc. B* 365: 593–603.

Moczek, A. P., et al. 2011. The role of developmental plasticity in evolutionary innovation. *Proc. R. Soc. B* 278: 2705–2713.

Morgan, K., et al. 2012. Multi locus analysis of *Pristionchus pacificus* on La Réunion Island reveals an evolutionary history shaped by multiple introductions, constrained dispersal events and rare out-crossing. *Mol. Ecol.* 21: 250–266.

Nijhout, H. F. 2003. Development and evolution of adaptive polyphenisms. *Evol. Dev.* 5: 9–18.

Ogawa, A., Bento, G., Bartelmes, G., Dieterich, C., and Sommer, R. J. 2011. *Pristionchus pacificus daf-16*/FOXO regulates dauer formation but not mouth form dimorphism. *Development* 138: 1281–1284.

Pfennig, D. 1990. The adaptive significance of an environmentally-cued developmental switch in an anuran tadpole. *Oecologia* 85: 101–107.

Pfennig, D. W., Wund, M. A., Snell-Rood, E. C., Cruickshank, T., Schlichting, C. D., and Moczek, A. P. 2010. Phenotypic plasticity's impacts on diversification and speciation. *Trend. Ecol. Evol.* 25: 459–467.

Rae, R., et al. 2008. Isolation of naturally associated bacteria of necromenic *Pristionchus* nematodes and fitness consequences. *J. Exp. Biol.* 211: 1927–1936.

Rechavi, O., Minevich, G., and Hobert, O. 2011. Transgenerational inheritance of an acquired small RNA-based antiviral response in *C. elegans*. *Cell* 147: 1248–1256.

Schlager, B., Röseler, W., Zheng, M., Gutierrez, A., and Sommer, R. J. 2006. HAIRY-like transcription factors and the evolution of the nematode vulva equivalence group. *Curr. Biol.* 16: 1386–1394.

Schlager, B., Wang, X. Y., Braach, G., and Sommer, R. J. 2009. Molecular cloning of a dominant roller mutant and establishment of DNA-mediated transformation in the nematode model *Pristionchus pacificus*. *Genesis* 47: 300–304.

Schlichting, C. D., and Pigliucci, M. 1998. *Phenotypic Evolution: A Reaction Norm Perspective*. Sinauer, Sunderland, MA.

Shine, R. 1989. Ecological causes for the evolution of sexual dimorphism: a review of the evidence. *Q. Rev. Biol.* 64: 419–461.

Shirayama, M., et al. 2012. piRNAs initiate an epigenetic memory of nonself RNA in the *C. elegans* germline. *Cell* 150: 65–77.

Snell-Rood, E. C., and Moczek, A. P. 2012. Insulin signaling as a mechanism underlying developmental plasticity: the role of *FOXO* in a nutritional polyphenism. *PLoS ONE* 7: e34857.

Sommer, R. J. 2009. The future of evo-devo: model systems and evolutionary theory. *Nat. Rev. Genet.* 10: 416–422.

Sommer, R. J., and Ogawa, A. 2011. Hormone signaling and phenotypic plasticity in nematode development and evolution. *Curr. Biol.* 21: R758–R766.

Sommer, R. J., Carta, L. K., Kim, S. Y., and Sternberg, P. W. 1996. Morphological, genetic and molecular description of *Pristionchus pacificus* n. sp. (Nematoda: Neodiplogastridae). *Fund. Appl. Nematol.* 19: 511–521.

Srinivasan, J., et al. 2002. A bacterial artificial chromosome-based genetic linkage map of the nematode *Pristionchus pacificus*. *Genetics* 162: 129–134.

Srinivasan, J., et al. 2003. An integrated physical and genetic map of the nematode *Pristionchus pacificus*. *Mol. Genet. Genomics* 269: 715–722.

Sternberg, P. W. 2005. Vulva development (June 25, 2005). In *The C. elegans Research Community (ed.)*. WormBook. (published online: doi: 10.1895/wormbook.1.6.1) http://www.wormbook.org.

Suzuki, Y., and Nijhout, H. F. 2006. Evolution of a polyphenism by genetic accommodation. *Science* 311: 650–652.

von Lieven, A. F. 2005. The embryonic moult in diplogastrids (Nematoda)—homology of developmental stages and heterochrony as a prerequisite for morphological diversity. *Zool. Anz.* 244: 79–91.

von Lieven, A. F., and Sudhaus, W. 2000. Comparative and functional morphology of the buccal cavity of Diplogastrina (Nematoda) and a first outline of the phylogeny of this taxon. *J. Zool. Syst. Evol. Res.* 38: 37–63.

Weller, A. M., Mayer, W. E., Rae, R., and Sommer, R. J. 2010. Quantitative assessment of the nematode fauna present on *Geotrupes* dung beetles reveals species-rich communities with a heterogeneous distribution. *J. Parasitol.* 96: 525–531.

West-Eberhard, M. J. 2003. *Developmental Plasticity and Evolution*. Oxford University Press, New York.

Yeates, G. W., Bongers, T., de Goede, R. G. M., Freckman, D. W., and Georgieva, S. S. 1993. Feeding habits in soil nematode families and genera—an outline for soil ecologists. *J. Nematol.* 25: 315–331.

Zheng, M., Messerschmidt, D., Jungblut, B., and Sommer, R. J. 2005. Conservation and diversification of Wnt signaling function during the evolution of nematode vulva development. *Nat. Genet.* 37: 300–304.

Downloaded from http://rspb.royalsocietypublishing.org/ on July 20, 2015

PROCEEDINGS OF THE ROYAL SOCIETY B

rspb.royalsocietypublishing.org

Research

Cite this article: Serobyan V, Ragsdale EJ, Sommer RJ. 2014 Adaptive value of a predatory mouth-form in a dimorphic nematode. *Proc. R. Soc. B* **281**: 20141334.
http://dx.doi.org/10.1098/rspb.2014.1334

Received: 9 June 2014
Accepted: 4 July 2014

Subject Areas:
evolution

Keywords:
developmental plasticity, feeding behaviour, fitness, polyphenism, *Pristionchus pacificus*, trade-off

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

Electronic supplementary material is available at http://dx.doi.org/10.1098/rspb.2014.1334 or via http://rspb.royalsocietypublishing.org.

Royal Society Publishing

Adaptive value of a predatory mouth-form in a dimorphic nematode

Vahan Serobyan[1], Erik J. Ragsdale[1,2] and Ralf J. Sommer[1]

[1]Department of Evolutionary Biology, Max Planck Institute for Developmental Biology, Spemannstraße 37, 72076 Tübingen, Germany
[2]Department of Biology, Indiana University, 1001 East 3rd Street, Bloomington, IN 47405, USA

Polyphenisms can be adaptations to environments that are heterogeneous in space and time, but to persist they require conditional-specific advantages. The nematode *Pristionchus pacificus* is a facultative predator that displays an evolutionarily conserved polyphenism of its mouthparts. During development, *P. pacificus* irreversibly executes either a eurystomatous (Eu) or stenostomatous (St) mouth-form, which differ in the shape and number of movable teeth. The Eu form, which has an additional tooth, is more complex than the St form and is thus more highly derived relative to species lacking teeth. Here, we investigate a putative fitness trade-off for the alternative feeding-structures of *P. pacificus*. We show that the complex Eu form confers a greater ability to kill prey. When adults were provided with a prey diet, Eu nematodes exhibited greater fitness than St nematodes by several measures, including longevity, offspring survival and fecundity when followed by bacterial feeding. However, the two mouth-forms had similar fecundity when fed ad libitum on bacteria, a condition that would confer benefit on the more rapidly developing St form. Thus, the two forms show conditional fitness advantages in different environments. This study provides, to our knowledge, the first functional context for dimorphism in a model for the genetics of plasticity.

1. Introduction

Developmental plasticity has been proposed to foster adaptation and rapid diversification by providing a ready source of selectable variation [1,2]. Polyphenisms in particular allow jumps to alternative adaptive peaks, including responses to environments that are heterogeneous in space and time, without disruptive selection on a particular morphology. To be both adaptive and evolutionarily stable, alternative forms must each be more favourable under a given set of environmental conditions [3]. Where alternative phenotypes arise from a single genotype, developmental outcomes are often finely tuned to the environment to optimize fitness, such as in response to seasonal changes [4], the presence of predators [5] or the availability of a given food source [6]. However, clear demonstrations of the benefits and costs of polyphenisms under different environmental conditions are still elusive. Separate developmental modules as allowed by a developmental switch [7] could allow diversifying selection on morphs [1,8], but the conditions favouring alternative morphs must be identified to test this principle.

Polyphenisms that allow alternative resource use are of particular interest for the relationship between plasticity and diversity. In contrast to alternative phenotypes that are costly responses to transient antagonistic pressures in the environment, resource polyphenisms enable niche partitioning and its associated character displacement within species [9]. The consequent divergence within species might then be followed by genetic assimilation or accommodation of optimized forms [10,11], possibly leading to morphological diversification [1,2,12,13]. Given the abundance of known resource polyphenisms, such plasticity may be a common diversifying force in nature [1,9,14]. Determining and quantifying fitness effects on alternative phenotypes can test their adaptive value and thus their ability to persist and diverge.

The nematode *Pristionchus pacificus*, an emerging model for comparative mechanistic studies of developmental traits [15], exhibits a morphological

© 2014 The Author(s) Published by the Royal Society. All rights reserved.

Downloaded from http://rspb.royalsocietypublishing.org/ on July 20, 2015

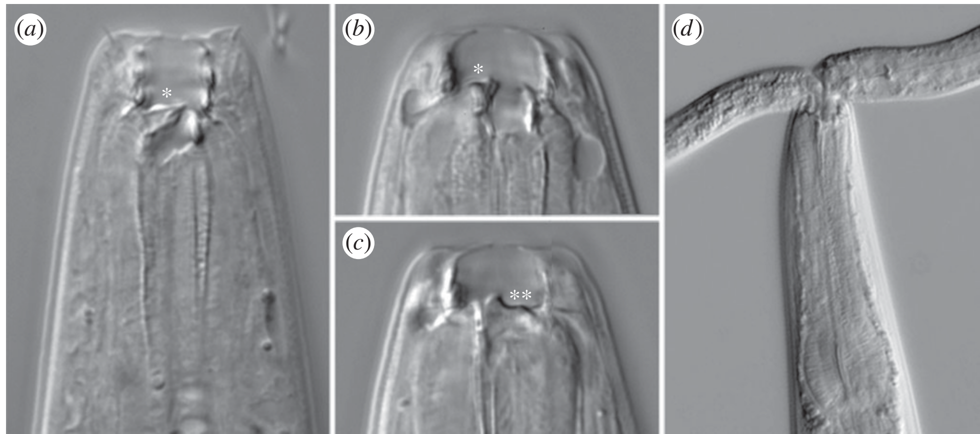


Figure 1. Mouth dimorphism and predation in *P. pacificus*. Movable teeth and a mouth dimorphism are both novelties of *P. pacificus* and other Diplogastridae and have enabled predation in this nematode family. (*a*) St form, with a single, triangular dorsal tooth (asterisk). (*b*) Eu form, with a claw-like dorsal tooth (asterisk). (*c*) Right sublateral focal plane of same individual in (*b*), showing a second, opposing tooth (double asterisks). Compared to the St form, the Eu form is more complex and highly derived with respect to outgroups that lack teeth or a mouth dimorphism. (*d*) Eu hermaphrodite of *P. pacificus* attacking a larva of *C. elegans*. Photograph in (*d*) by Dan Bumbarger.

dimorphism that may correlate with variable food resources [16]. The feeding-structure dimorphism of *P. pacificus* consists of contrasting mouthparts in the adult stage (figure 1*a–c*). The dimorphism results from an irreversible decision during larval development, as mouthparts are produced during the final moult of the nematode life cycle. Both mouth-forms possess a dorsal tooth that has enabled predation (figure 1*d*) and, like the dimorphism, is a novelty of the nematode family Diplogastridae [17]. However, one of the forms, the eurystomatous (Eu, 'wide-mouthed') form, is equipped with even more complex structures, including an opposing tooth and a heavily serrated plate of denticles [18]. The Eu form is highly derived with respect to the closest outgroups that lack teeth, whereas the alternative, stenostomatous (St, 'narrow-mouthed') form is more reminiscent of outgroups in terms of mouth complexity and shape [9]. With such a dimorphism, this model system has been amenable to advances in understanding the genetic regulation of plasticity, including neuroendocrine signalling [19] and a developmental switch from a new gene [7].

The variable environments in which *P. pacificus* and other diplogastrids proliferate, especially the cadavers of host insects [20,21], may favour a resource polyphenism. These environments are rich and dynamic, being rapidly colonized by various bacteria [22], fungi and other nematodes [23]. Consistent with conditional regulation in response to such an environment, the dimorphism of *P. pacificus* is influenced by signals that indicate resource availability. Starvation, specifically the deprivation of bacterial food, is one cue that triggers the formation of the Eu form [19]. The presence of conspecifics, which is sensed by small molecules, also induces development of the Eu form [24,25]. By responding to crowding, the dimorphism may result in a frequency-dependent switch to alternative food sources [6]. Specifically, this switch might allow better predation on other nematodes, possibly even cannibalism, which has been anecdotally observed in *P. pacificus* (V. Serobyan, E. J. Ragsdale 2013, unpublished data).

Despite its conditional regulation, the dimorphism is apparently also stochastically regulated. Both mouth-forms are consistently present in populations under all tested experimental conditions. Moreover, the majority of wild *P. pacificus* populations examined have a mouth-form frequency biased towards the Eu form (i.e. more than 50% of a population) when fed exclusively on bacteria [7], a diet that in nematodes requires neither teeth nor dimorphism. The production of both forms in every generation thus suggests a partial bet-hedging strategy that is based on probabilities for enhanced fitness under variable conditions [26]. The ephemeral environments *P. pacificus* inhabits are unpredictable, presumably occurring with irregular frequency with respect to the life cycle of the nematode. Therefore, a dimorphism with mixed regulation may be advantageous for exploiting such temporally uncertain and variable niches, although precisely how the two feeding-forms use resources differently was heretofore unknown.

As a first response to this problem, the potential for a fitness trade-off in the dimorphism of *P. pacificus* was recently demonstrated. Specifically, the St form was found to develop to maturity faster on a bacterial diet, presumably owing to its simpler morphology and accompanying physiological differences [24]. Specifically, St animals proceed through postembryonic development about 6 h faster than Eu animals, resulting in a head start in reproduction for the St form in a population. Given the nematodes' short generation time of 3–4 days, this difference in developmental rate gives the St form of *P. pacificus* a significant advantage when feeding on bacteria, a benefit that would be particularly exaggerated over the several generations possible on ephemeral resources. However, because individuals of both forms are prolific on a bacterial diet, some fitness advantage for the Eu form would be required to ensure the persistence of a dimorphism. Alternative food sources, including other nematodes, could offer the necessary conditions to favour the Eu form. The ability to feed on other nematodes is a commonly observed phenomenon in *Pristionchus* and other Diplogastridae (figure 1*d*), although it was previously unknown whether this ability differs between the two forms. Because of its wide mouth and opposing teeth, the Eu form could be predicted by analogy to be the superior predator; to the best of our knowledge, however, this precept has never been tested.

Fitness tests are especially feasible in *P. pacificus* owing to the hermaphroditic mode of reproduction in this species. Hermaphrodites (XX morphological females) can also facultatively outcross, as males (X0 animals) are occasionally produced by

Downloaded from http://rspb.royalsocietypublishing.org/ on July 20, 2015

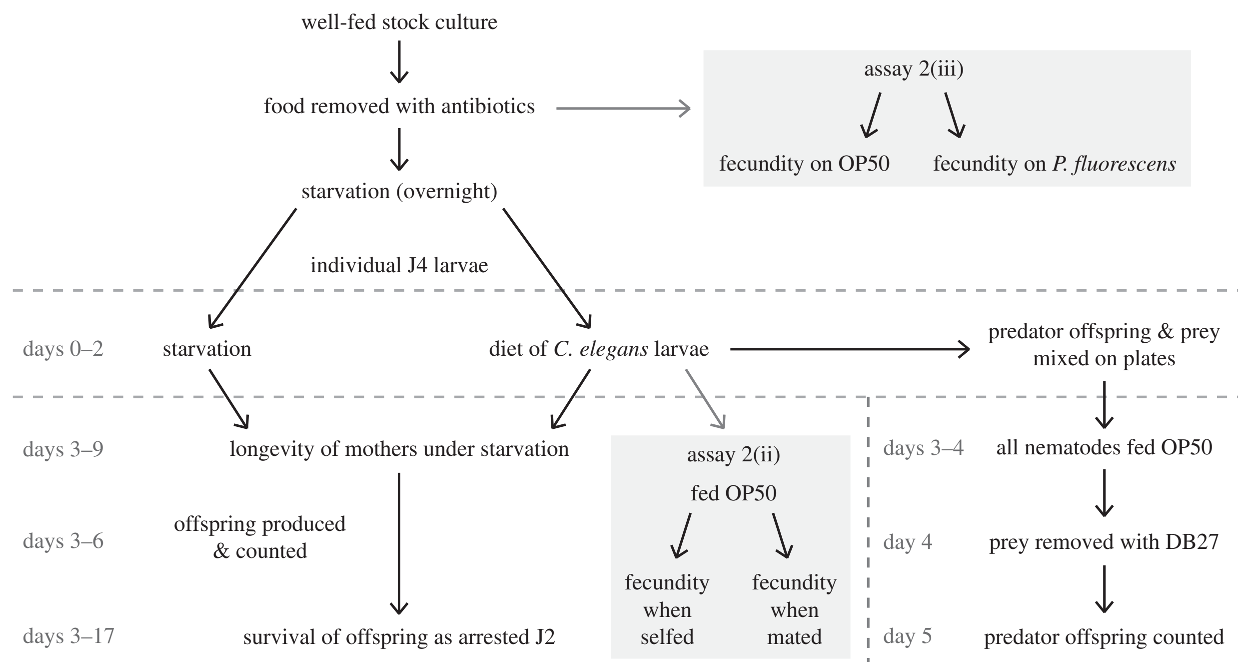


Figure 2. An assay for fitness differences between Eu and St mouth-forms fed on prey. Figure corresponds to methods described in §2b of text, with alterations shown for assays in 2(ii) and 2(iii) as described in the electronic supplementary material.

meiotic X-chromosome non-disjunction [27]. This reproductive strategy makes *P. pacificus* amenable to direct measurements of fitness of either mated or unmated individuals in response to experimental conditions. Here, by developing experimental assays involving predators and prey, we have tested the functional and fitness advantages of the Eu form of *P. pacificus*.

2. Material and methods

To test the adaptive value of alternative forms, we measured their respective functional and fitness advantages. First, as a test of function of form-specific morphologies, we assayed the ability of each form to kill prey. Second, we measured the fitness of alternative forms that were fed prey as adults (figure 2). Third, we measured the fitness of forms when fed prey and then afterwards allowed to feed on bacteria, which we identified as a much more nutritional food source then prey. Finally, to complement an earlier study on developmental times on a bacterial diet [24], we tested for fitness advantages of the alternative forms in the presence of abundant bacteria.

In the following, we describe the methodology of the predatory assay §2a and the fitness differences on prey diet §2b. Other parts of the methodology, i.e. (i) nematode cultivation and phenotypes, (ii) fecundity on a heterogeneous adult diet, (iii) differential fecundity on a bacterial diet, and (iv) statistical analyses are found in the electronic supplementary material.

(a) Predation assay

To test for form-specific differences in the ability to prey on other nematodes, we developed an assay to quantify components of the predatory behaviour of *P. pacificus*. To prepare *P. pacificus* for this assay, nematodes were washed from stock-culture plates, rinsed twice in water with antibiotics (50 μg ml^{-1} ampicillin) to remove residual food, starved overnight on nematode growth medium (NGM) agar plates without cholesterol or peptone and kept at 20°C, and then placed at 12°C the next morning to prevent further starvation before their use in assays that day. Young adults (i.e. carrying 0–2 eggs) were subjected to the assays.

Prey for the predation assay consisted of first-stage (L1) larvae of the nematode *Caenorhabditis elegans*. This species is a member of a group of nematodes, 'Rhabditidae', that *P. pacificus* often encounters in the wild. Rhabditidae are widespread in ephemeral habitats, which both rhabditid and diplogastrid nematodes can rapidly colonize [28]. Consequently, *P. pacificus* has been observed to feed on other nematodes of Rhabditidae, including *Pelodera* spp. (data not shown), which are commonly found on the same beetle hosts of *Pristionchus* spp. [23]. Furthermore, *C. elegans* exhibits very similar external morphology to known prey species [29,30], secreted chemical profiles of Rhabditina (including Rhabditidae) are highly conserved [31], and locomotive behaviour, including responses to mechanical stimuli, is stereotypic for nematodes of Rhabditidae. Cultures of *C. elegans* strain N2 were maintained on NGM agar with OP50 as described above for *P. pacificus*. To produce larvae for assays, plates of *C. elegans* were bleached to release eggs and remove all larvae and adults, following standard protocols [32]. After rinsing eggs into water, the density of eggs was estimated by agitating the volume and counting eggs dispensed in two 5 μl drops. Eggs were then dispensed onto NGM plates with no food, cholesterol or peptone and at a standard density of 12 000 eggs per plate. After incubating plates overnight at 20°C, hatched L1 larvae emerged on plates. Prey larvae, although active, were developmentally arrested at this stage, ensuring very little variation in prey age or size within or among replicates and experiments.

For each assay, a single *P. pacificus* individual was gently transferred to a prey plate and monitored with a Zeiss Discovery V.12 stereomicroscope. The assay was started after the predator was allowed to recover from handling, namely upon detection of predator locomotion. Recovery time did not differ between mouth-forms (Kruskal–Wallis ANOVA, $p > 0.2$; data not shown). Predators were placed near the centre of the plate, throughout which prey were distributed by their own dispersal behaviour. In the assay, the behaviour of *P. pacificus* was scored for 'encounters', 'attacks' and 'kills'. An encounter was recorded when the anterior tip (lips) of the predator made perpendicular contact with a *C. elegans* larva; an attack was recorded if, during an encounter, anterior body movement of the predator ceased and pharynx pumping initiated, which included deployment of the dorsal tooth; a kill was recorded if the body wall of the prey was ruptured, as evident from the release of body pressure or

Downloaded from http://rspb.royalsocietypublishing.org/ on July 20, 2015

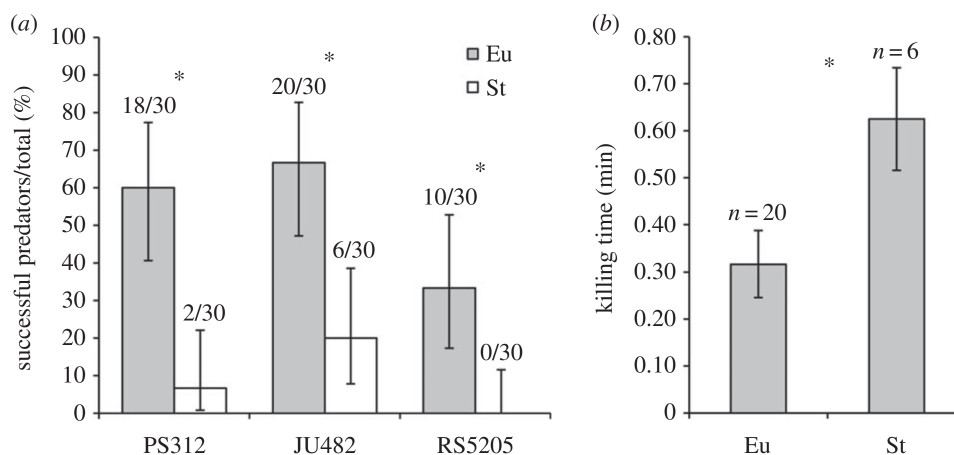


Figure 3. Predatory (killing) ability in Eu versus St hermaphrodites of *P. pacificus*. (*a*) The percentage of assayed individuals able to kill a prey larva within 10 min, as tested for three strains of *P. pacificus*. In all assayed strains, the Eu form was the more successful predator (*$p < 0.05$, χ^2-test). Absolute numbers of successful predators are given above bars. Whiskers represent a 95% binomial confidence interval. (*b*) The time required for hermaphrodites *P. pacificus* strain JU482 to kill their prey. Individuals tested were those successful in the predation assay. Time was measured from the onset of the final attack until rupture of the prey body cuticle. Whiskers represent the standard error. *$p < 0.05$, Student's *t*-test.

contents from prey. In the case of a kill, the time period from the start of the fatal attack to the rupture of prey cuticle was measured. The assay was performed for 10 min, unless a prey item was successfully killed, at which time the assay was ended. Predatory ability for each strain and mouth-form combination was assessed by the proportion of tested individuals able to successfully kill a prey item. Three strains of *P. pacificus* were assayed for predatory ability: (i) PS312, the laboratory reference strain ('California'); (ii) JU482 (Japan), which was previously noted to be a relatively successful predatory strain; and (iii) RS5205 (South Africa), which has a ratio of mouth-forms close to 1 : 1 in well-fed culture [7]. For each strain, 30 individuals of each mouth-form were assayed.

(b) Fitness differences on a prey diet

To measure fitness differences between forms when offered a diet of prey, we developed an assay to allow potential predators to feed on only prey at early adulthood (figure 2). In this experiment, prey plates were prepared as for the predation assay, except that prey was administered at a density of 8000 *C. elegans* larvae per plate, and thus all potential predators were able to feed ad libitum. Strain RS5205 of *P. pacificus* was selected as the predatory strain, owing to the likelihood of selecting either mouth-form before adult morphology could be determined.

To start the experiment, 140 J4 (pre-adult) stage larvae of *P. pacificus* were transferred to individual prey plates and maintained at 20°C. Predators were kept on this feeding regimen for 2 days (days 0–1 of assay), during which the predators began expressing one of the two mouth-forms and would be able to acquire nutrition differentially as enabled by their feeding morphologies. As a control, 140 J4 *P. pacificus* larvae were transferred to identically prepared NGM double-agar (3.4%) plates with no prey and no bacteria. After this 2-day period (day 2), *P. pacificus* mothers from both types of plates were checked for their mouth phenotype and transferred to individual wells of a 96-well plate, with each well containing 250 µl S-medium with antibiotics.

The first fitness measure taken in this experiment was the fecundity of Eu and St *P. pacificus* hermaphrodites following a prey diet. All larvae and embryos with dividing cells were counted as offspring; any non-dividing embryos were considered dead eggs and not included in counts. After removal from prey and control (starvation) plates, hermaphrodites were transferred daily to new individual wells with S-medium, and screened for offspring until 2 days after removal from plates (day 4), by which time all hermaphrodites in wells stopped laying eggs. In this manner, offspring produced on each day could be counted separately. To count offspring produced on prey plates during feeding (days 0–1, pooled as day 1), predators needed to be separated from prey, as the similar body shapes of the two species rendered distinction of *P. pacificus* larvae among thousands of prey larvae impossible. To accomplish this, we first transferred all individuals on prey plates to NGM plates with OP50, letting both species recover from starvation for 2 days (days 3–4). Following this recovery period, we recruited a pathogenic strain of bacteria, *Bacillus thuringiensis* strain DB27, that after 24 h of exposure is completely lethal to *C. elegans* but not at all to *P. pacificus* [33,34]. One day after administering DB27 to plates in a quantity of 60 µl in lysogenic broth per plate (day 5), *P. pacificus* offspring could be readily distinguished and counted. Offspring produced on control plates, which contained no prey, were simply screened 1 day after removing *P. pacificus* mothers from plates.

Second, we assayed the longevity of Eu and St *P. pacificus* hermaphrodites following a prey diet. To do this, we continued the daily transfer of hermaphrodites, already assayed for fecundity, to new wells containing S-medium (with antibiotics) until 7 days after removal from plates (day 9). At that time, the only three survivors were additionally checked for their ability to produce offspring if fed and mated, although they had stopped producing self-offspring at least 4 days earlier. These individuals were placed along with one adult male onto NGM agar plates with OP50 and let to feed and mate indefinitely.

Third, to obtain a measure of quality for the offspring of prey-fed mothers, we assayed the survival of offspring as developmentally arrested larvae. In this assay, a sample of 96 J2 hatchlings produced the first day after removal from prey plates were taken for each test group. Larvae were placed in individual wells containing 250 µl S-medium (with antibiotics) and monitored at intervals of 12 h until dead. Death was scored if larvae were no longer motile, even after mild agitation of wells.

3. Results

(a) Eurystomatous animals have a functional advantage in predation

In spite of their morphological differences, the differential function of the two forms of *P. pacificus*, or of other dimorphic diplogastrid nematodes, was previously unknown. In this study, three strains of *P. pacificus* were assayed for their ability to kill other nematodes provided as prey (figure 3*a*). In each

Downloaded from http://rspb.royalsocietypublishing.org/ on July 20, 2015

Table 1. Brood sizes of *P. pacificus* (RS5205) hermaphrodites that were starved or prey-fed as adults. (On day 0, virgin J4 (pre-adult) hermaphrodites were introduced to the experimental feeding regime (figure 2). On day 2, hermaphrodites were removed from experimental feeding regimen to wells for starvation. Offspring were counted throughout reproductive lifetime of hermaphrodites. Values are given in the form: median (lower quartile-upper quartile).)

	day 1	day 2	days 3–4	total
Eu starved	1 (1–3)	10 (7–12)	0 (0–1)	12 (10–16)
St starved	6 (3–9)	1 (0–3)	1 (0–4)	11 (8–13)
Eu prey-fed	0 (0–2)	7 (5–9)	4 (1–6)	13 (10–16)
St prey-fed	8 (2–11)	3 (1–5)	0	10 (6–15)

strain, the Eu form was able to kill significantly more often than the St form (PS312, $\chi^2 = 8.40$, $p < 0.005$; JU482, $\chi^2 = 6.83$, $p < 0.005$; RS5205, $\chi^2 = 4.35$, $p < 0.05$). At the level of individuals, therefore, the Eu form was functionally a more successful predator. To test whether predatory ability could be owing to differences in hunting behaviour, the rates of encounters per minute and encounters per attack were compared between forms of strain JU482, the most successful predators of the strains tested. No differences were detected between mouth-forms with either successful or unsuccessful predatory behaviour (Kruskal–Wallis, $p > 0.1$; electronic supplementary material, figure S1). Nevertheless, in successful acts of predation, the time required to kill (figure 3*b*) was found to be significantly greater for the St form (KW-$H_{1,26} = 5.80$, $p < 0.05$), such that the Eu form could kill in half the time needed for the St form. Taken together, our assay showed the Eu form to be a superior predator. This ability was not attributed to locating prey, but to the efficiency of turning an attack into a kill.

(b) Differential fecundity on a prey diet

To test whether the functional advantage of the Eu form translated to a greater adaptive value, we tested for fitness differences between forms following a diet enriched in live prey (table 1). When potential predators were fed ad libitum on *C. elegans* larvae, there were no differences in the total brood size produced after feeding by the Eu and St forms or test (prey-fed) and control (starved) groups (KW-$H_{3,145} = 7.96$, $p = 0.07$), which had a median brood size of 12. However, differences were detected in the daily brood size in both test and control groups: the St form produced more offspring the first day after feeding (KW-$H_{3,145} = 53.9$, $p < 0.0005$), whereas the Eu form produced more on the second day (KW-$H_{3,145} = 70.7$, $p < 0.0005$) and on the third and fourth days (KW-$H_{3,145} = 35.2$, $p < 0.0005$). This result confirms the faster developmental time to maturity for the St form [24].

Although the Eu and St forms showed no fecundity differences when starved after a prey diet, we tested in a separate experiment whether prey-fed hermaphrodites could produce more offspring when reintroduced to bacterial food (figure 4*a*). In contrast to the starved hermaphrodites, which showed no difference between forms (KW-$H_{1,21} = 0.6$, $p = 0.44$), the fecundity of Eu hermaphrodites was

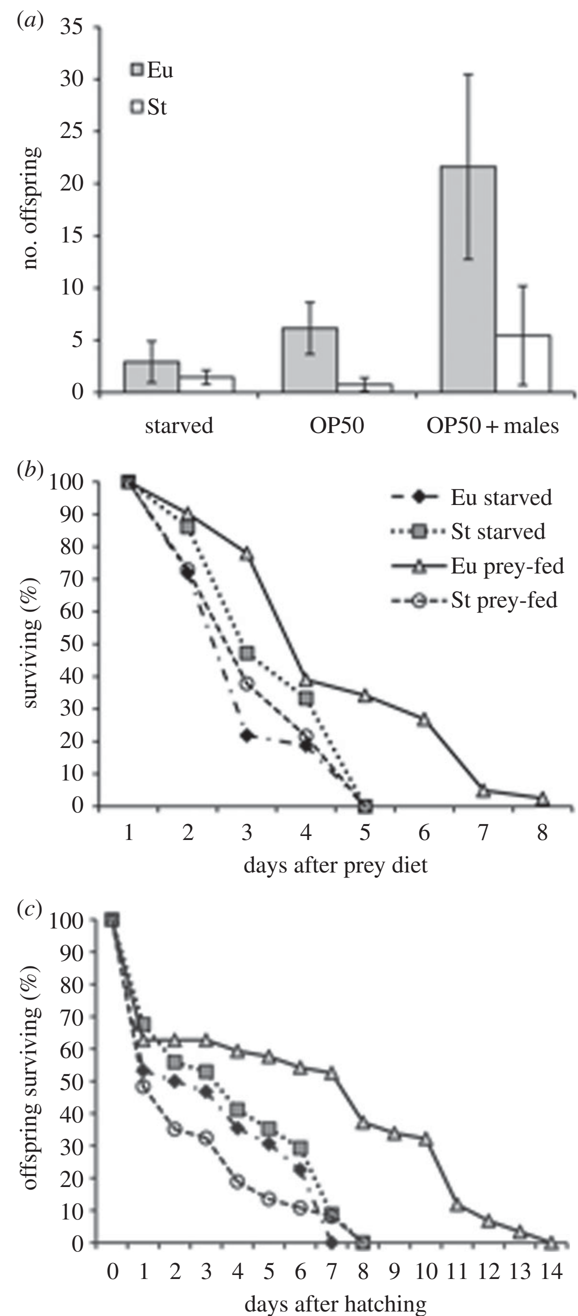


Figure 4. Fitness measures of Eu and St hermaphrodites of *P. pacificus* RS5205 after feeding on prey. (*a*) Fecundity (brood size) of hermaphrodites after a prey diet and when also fed bacteria (OP50) or fed bacteria and mated (OP50 + males). Whiskers represent the standard error. *$p < 0.05$, Kruskal–Wallis. (*b*) Longevity of starved and prey-fed hermaphrodites. Individuals were introduced as J4 (pre-adult) larvae to starvation or prey plates on day 0. On day 8, the last surviving hermaphrodites were removed from assay to test their fertility, for which two out of three were positive. (*c*) Survival of arrested J2 (first post-eclosion stage) offspring produced by starved or prey-fed hermaphrodites.

significantly higher than that of St hermaphrodites when reintroduced to bacteria (KW-$H_{1,32} = 10.4$, $p < 0.005$). Fecundity experiments thus suggest that an adult diet of prey is challenging to both mouth-forms, but that when reintroduced to a nutritionally rich food source, namely bacteria, the Eu form recovered better than the St form.

When prey-fed hermaphrodites were introduced to both bacterial food and males, Eu hermaphrodites also produced more offspring than St hermaphrodites (KW-$H_{1,31} = 8.8$,

rspb.royalsocietypublishing.org *Proc. R. Soc. B* **281**: 20141334

Downloaded from http://rspb.royalsocietypublishing.org/ on July 20, 2015

$p < 0.005$), indicating that the nutritional challenge of a prey diet affected self-sperm production in both forms but was less detrimental to egg production in the Eu form than in the St form. Furthermore, hermaphrodites of the Eu form produced more offspring when mated versus not mated (KW-$H_{1,33} = 5.8$, $p < 0.05$), although the St form showed no such increase when mated (KW-$H_{1,30} = 0.8$, $p = 0.36$). Therefore, the nutritional challenge to the more resilient Eu form can be explained by the effect of a poor diet on self-sperm production.

Finally, when fed only bacteria, the two mouth-forms showed no fecundity differences on either OP50 ($T_{58} = 0.998$, $p = 0.32$; electronic supplementary material, figure S2) or a naturally associated strain of *P. fluorescens* ($T_{56} = -0.784$, $p = 0.44$; electronic supplementary material, figure S2), indicating that both forms were equally efficient in converting energy acquired from bacteria to reproductive output when fed ad libitum. This finding yields two major conclusions. First, it shows that bacteria are a far better food resource than prey for either mouth-form (figure 4*a* and table 1). This confirms that the Eu form is adaptive as a stress-resistant form in conditions of low bacteria but high prey availability. Second, the ability of the St form to produce as many offspring as the Eu form, when combined with a shorter generation time for the St form [24], could translate to higher fitness for that form in a bacteria-rich environment.

(c) Survival differences of prey-fed mothers and their offspring

In another measure of fitness, we assayed the longevity of hermaphrodites fed on prey (figure 4*b*). Prey-fed Eu hermaphrodites survived significantly longer than prey-fed St, starved Eu, and starved St hermaphrodites (log-rank, $\chi^2_3 = 38.9$, $p < 0.0005$), further indicating the Eu form to be more resilient to the absence of bacterial food but presence of nematode prey. By contrast, the latter three groups did not differ from each other (log-rank, $\chi^2_2 = 3.66$, $p = 0.16$). Because in this assay, longer lived hermaphrodites outlived their capacity to produce self-offspring, at the end of the assay we tested whether the surviving hermaphrodites were still fertile by providing them with bacterial food and a mate. Seven days following their removal from plates, including 4 days without laying eggs, two of the three tested survivors produced hatching offspring, one of them even producing over 40 offspring (data not shown).

Finally, a measure of the quality of offspring produced by mothers fed on prey was taken by measuring the survival of hatched offspring that were themselves never fed (figure 4*c*). Larvae remained arrested in the first post-eclosion (J2) stage, presumably persisting on energy reserves provisioned by the mother. In this assay, the offspring of prey-fed Eu hermaphrodites survived significantly longer than those of prey-fed St, starved Eu and starved St hermaphrodites (log-rank, $\chi^2_3 = 44.0$, $p < 0.0005$). By contrast, the latter three groups did not differ from each other (log-rank, $\chi^2_2 = 3.66$, $p = 0.27$). The survival of arrested young larvae thus revealed Eu mothers to be more fit than St mothers after a diet of prey.

4. Discussion

In spite of mechanistic advances towards understanding the regulation and evolution of a developmental dimorphism in *P. pacificus* [7,19,25], the ecological context for morphological differences in this model organism was heretofore untested. Furthermore, the precise costs and benefits of resource polyphenisms under different environments have been generally difficult to determine in nature. Here, we have used an experimental approach in a genetically and environmentally controlled setting to determine the condition-dependent advantages of the more complex of the two feeding-forms. This study thus provides, to our knowledge, the first functional and evolutionary context for plasticity in *P. pacificus*, and it establishes a laboratory model for interpreting costs and benefits of a polyphenism as it responds to cues in the wild.

(a) A superior predatory form in *Pristionchus pacificus*

Following our development of a predation assay, we have shown with empirical evidence that the Eu form confers an advantage over the St form in this mode of feeding. Given a set length of time and abundant nematode larvae, more Eu than St individuals were able to kill their prey. In cases where the St form was able to kill a prey item, the predator required more time to complete the task, indicating either a difference in pharyngeal behaviour, such as rapidity of tooth movement or morphological limitations. A functional explanation for the superior killing ability of the Eu form would be sufficient based on morphological differences alone. First, the hooked subventral tooth of the Eu form may enable that form to engage the body wall of its prey, whereas the dorsal tooth is deployed opposite to slice the substrate, as described for the diplogastrid *Tylopharynx foetida* when fed on fungal spores [35]. By contrast, the St form lacks this tooth and must therefore rely on suction to keep its prey engaged. Second, the wider mouth of the Eu form may simply allow a substrate with a given elasticity or curvature to enter deeper into the mouth cavity. Because differences between the two forms in their killing ability are now established, testing differences at a neurological level may also be ultimately possible, given the reconstructed wiring for the pharyngeal nervous system in *P. pacificus* as a reference [36].

(b) Condition-dependent fitness advantages

In the presence of prey nematodes, the Eu form of *P. pacificus* showed higher fitness than the St form by several measures: (i) fecundity when offered bacteria as food after feeding on prey; (ii) longevity; and (iii) survival of starved, developmentally arrested offspring. By contrast, survival assays showed no advantage for the St form over starved individuals of either form, suggesting a poor ability to convert prey as a food resource to reproductive output. Therefore, under conditions restricted in bacterial resources but replete with prey, the Eu form was shown to confer the higher adaptive value to *P. pacificus*.

The Eu form was more prolific when fed a heterogeneous diet as adults, although neither morph showed an advantage when fed on prey and subsequently starved. Additionally, lifetime brood sizes of virgin, prey-fed hermaphrodites were small relative to those fed exclusively on bacteria, indicating prey to be an inferior food resource to bacteria for both mouth-forms. In our assay, an adult diet of only prey elicited a starvation-like response, recalling the 'adult reproductive diapause' of *C. elegans*, whereby hermaphrodites starved from the fourth larval stage onwards experience a similar reduction of fecundity [37]. In *C. elegans*, this reduction of

Downloaded from http://rspb.royalsocietypublishing.org/ on July 20, 2015

brood size reflects starvation-induced shrinkage of the germline, presumably to divert nutrition to the few developing eggs produced [38]. In the wild, however, it is more likely that *P. pacificus* encounters both bacterial and animal food sources [22,23], albeit in variable abundances. Consequently, assays of reproductive recovery when fed with prey followed by bacteria indicate a fitness benefit for the Eu form on a heterogeneous food resource. Specifically, the superior ability of Eu individuals to recover from a nutritionally poor diet of only prey suggests the Eu form to be more stress-resistant than the St form. We speculate, therefore, that in environments where bacteria are present but limited, the higher fecundity of the Eu form when supplemented with prey confers an adaptive advantage for that form.

In contrast to the small brood sizes of virgin hermaphrodites fed on prey, Eu hermaphrodites that were subsequently fed bacteria and mated were more fecund. This result suggests that in starved or prey-fed hermaphrodites, the number of available self-sperm is reduced, possibly as a sacrifice for provisioning the few eggs that can be fertilized and laid [38]. Restricted food conditions may therefore favour the Eu form, at least under some conditions as tested here, because it can exhibit greater fitness on a mixed diet. The latter consequence would provide an advantage of the Eu form for exploiting co-occurring microbivorous species, which compete for the same diminishing bacterial food source, as potential prey.

The survival curves for both prey-fed mothers and their offspring show the advantage of Eu individuals and their offspring to persist under prey-enriched conditions, presumably owing to greater energy reserves. The longer survival of arrested larvae, if similar in function to the ageing-suspending, stress-resistant L1 arrested larvae of *C. elegans* [39,40], would, in principle, also impart a greater probability of reaching maturity and reproducing. Enhanced survival of young *P. pacificus* larvae would allow their more successful dispersal to new bacteria-rich habitats, such as with the death of their insect hosts.

(c) A fitness trade-off supports maintenance of alternative forms

The conditional advantages of the Eu form, demonstrated here on a regimen of predatory feeding, complement the recent finding that the St form reaches the age of maturity faster on a bacterial diet [24]. Consistent with that finding, hermaphrodites that mature from the pre-adult stage on either a diet of prey or under starvation conditions, the St was reproductively active sooner than the Eu form. The absence of a fecundity advantage for either form when fed ad libitum on bacteria supports an advantage for the St form under conditions rich with that food source. The presence of environmentally dependent fitness optima for the two forms suggests a fitness trade-off, satisfying the predicted conditions for the persistence of a discontinuous dimorphism in evolution [3]. The equilibrium of forms might expectedly change in populations as an adaptation to a particular host or microhabitat [41], and a diversity of ratios has indeed been observed in *P. pacificus* [7]. However, the persistence of conditions benefitting both forms would favour a dimorphism, provided that the cost of maintaining plasticity is not itself prohibitive [42].

This study complements discussions on the adaptive significance of alternative phenotypes as the result of developmental switches. Previous analyses of polyphenisms have predicted fitness advantages by inferring costs and benefits of alternative forms [43–46]. In examples analogous to the predatory feeding dimorphism of *P. pacificus*, the density-dependent induction of predatory or cannibalistic morphs in ciliates [47,48], rotifers [49], *Ambystoma* salamanders [50–52] and spadefoot toads [6] have shown in particular how plasticity allows a rapid adaptive response that includes novel feeding strategies. However, the measurements of direct fitness that are required to test the adaptive value of alternative forms, specifically in the absence of heritable and environmental variation, are difficult to achieve in many systems. By testing the potential trade-off of a resource polyphenism in a hermaphroditic laboratory model, we have distinguished such adaptive values. The inferred trade-off thus suggests evolutionary advantages for specific genetic regulators of dimorphism as have been identified in *P. pacificus*. Furthermore, support for a persistent dimorphism suggests its macroevolutionary potential to buffer further adaptive variability [1,53]. In such a case, the adaptive significance of the mouth dimorphism would be of central importance for buffering the diversity of feeding morphology seen in *Pristionchus* and other dimorphic Diplogastridae [18,54,55].

Acknowledgements. We are thankful to Andreas Weller, who established an early protocol for the predation assay that we developed for this study. We also thank Robbie Rae and Vladislav Susoy for their advice on the project, and we thank Cameron Weadick for his insightful suggestions on the manuscript. V.S. and E.J.R. designed the research; V.S. performed the research; E.J.R., V.S. and R.J.S. wrote the manuscript.

Funding statement. We gratefully acknowledge the Alexander von Humboldt Foundation for the support of a fellowship awarded to E.J.R.

References

1. West-Eberhard MJ. 2003 *Developmental plasticity and evolution*. Oxford, UK: Oxford University Press.
2. West-Eberhard MJ. 2005 Developmental plasticity and the origin of species differences. *Proc. Natl Acad. Sci. USA* **102**, 6543–6549. (doi:10.1073/pnas.0501844102)
3. Moran NA. 1992 The evolutionary maintenance of alternative phenotypes. *Am. Nat.* **139**, 971–989. (doi:10.1086/285369)
4. Brakefield PM, Reitsma N. 1991 Phenotypic plasticity, seasonal climate and the population of *Bicyclus* butterflies (Satyridae) in Malawi. *Ecol. Entomol.* **16**, 291–303. (doi:10.1111/j.1365-2311.1991.tb00220.x)
5. Stibor H. 1992 Predator induced life-history shifts in a freshwater cladoceran. *Oecologia* **92**, 162–165. (doi:10.1007/BF00317358)
6. Pfennig DW. 1990 The adaptive significance of an envrionmentally-cued developmental switch in an anuran tadpole. *Oecologia* **85**, 101–107. (doi:10.1007/BF00317349)
7. Ragsdale EJ, Müller MR, Rödelsperger C, Sommer RJ. 2013 A developmental switch coupled to the evolution of plasticity acts through a sulfatase. *Cell* **155**, 922–933. (doi:10.1016/j.cell.2013.09.054)
8. West-Eberhard MJ. 1989 Phenotypic plasticity and the origins of diversity. *Annu. Rev. Ecol. Syst.* **20**, 249–278. (doi:10.1146/annurev.es.20.110189.001341)

Downloaded from http://rspb.royalsocietypublishing.org/ on July 20, 2015

9. Smith TB, Skúlason S. 1996 Evolutionary significance of resource polymorphisms in fishes, amphibians, and birds. *Annu. Rev. Ecol. Syst.* **27**, 111–133. (doi:10.1146/annurev.ecolsys.27.1.111)
10. Waddington CH. 1953 Genetic assimilation of an acquired character. *Evolution* **7**, 118–126. (doi:10.2307/2405747)
11. Suzuki Y, Nijhout HF. 2006 Evolution of a polyphenism by genetic accommodation. *Science* **311**, 650–652. (doi:10.1126/science.1118888)
12. Gomez-Mestre I, Buchholz DR. 2006 Developmental plasticity mirrors differences among taxa in spadefoot toads linking plasticity and diversity. *Proc. Natl Acad. Sci. USA* **103**, 19 021–19 026. (doi:10.1073/pnas.0603562103)
13. Ledon-Rettig CC, Pfennig DW, Nascone-Yoder N. 2008 Ancestral variation and the potential for genetic accommodation in larval amphibians: implications for the evolution of novel feeding strategies. *Evol. Dev.* **10**, 316–325. (doi:10.1111/j.1525-142X.2008.00240.x)
14. Pfennig DW, McGee M. 2010 Resource polyphenism increases species richness: a test of the hypothesis. *Phil. Trans. R. Soc. B* **365**, 577–591. (doi:10.1098/rstb.2009.0244)
15. Sommer RJ, McGaughran A. 2013 The nematode *Pristionchus pacificus* as a model system for integrative studies in evolutionary biology. *Mol. Ecol.* **22**, 2380–2393. (doi:10.1111/mec.12286)
16. Kiontke K, Fitch DHA. 2010 Phenotypic plasticity: different teeth for different feasts. *Curr. Biol.* **20**, R710–R712. (doi:10.1016/j.cub.2010.07.009)
17. Sudhaus W, Fürst von Lieven A. 2003 A phylogenetic classification and catalogue of the Diplogastridae (Secernentea, Nematoda). *J. Nematode Morphol. Syst.* **6**, 43–90.
18. Fürst von Lieven A, Sudhaus W. 2000 Comparative and functional morphology of the buccal cavity of *Diplogastrina* (Nematoda) and a first outline of the phylogeny of this taxon. *J. Zool. Syst. Evol. Res.* **38**, 37–63. (doi:10.1046/j.1439-0469.2000.381125.x)
19. Bento G, Ogawa A, Sommer RJ. 2010 Co-option of the hormone-signalling module dafachronic acid-DAF-12 in nematode evolution. *Nature* **466**, 494–497. (doi:10.1038/nature09164)
20. Herrmann M, Mayer WE, Sommer RJ. 2006 Nematodes of the genus *Pristionchus* are closely associated with scarab beetles and the Colorado potato beetle in Western Europe. *Zoology* **109**, 96–108. (doi:10.1016/j.zool.2006.03.001)
21. Herrmann M, Mayer WE, Hong RL, Kienle S, Minasaki R, Sommer RJ. 2007 The nematode *Pristionchus pacificus* (Nematoda: Diplogastridae) is associated with the Oriental beetle *Exomala orientalis* (Coleoptera: Scarabaeidae) in Japan. *Zool. Sci.* **24**, 883–889. (doi:10.2108/zsj.24.883)
22. Rae R, Riebesell M, Dinckelacker I, Wang Q, Herrmann M, Weller AM, Dieterich C, Sommer RJ. 2008 Isolation of naturally associated bacteria of necromenic *Pristionchus* nematodes and fitness consequences. *J. Exp. Biol.* **211**, 1927–1936. (doi:10.1242/jeb.014944)
23. Weller AM, Mayer WE, Rae R, Sommer RJ. 2010 Quantitative assessment of the nematode fauna present on *Geotrupes* dung beetles reveals species-rich communities with a heterogeneous distribution. *J. Parasitol.* **96**, 525–531. (doi:10.1645/GE-2319.1)
24. Serobyan V, Ragsdale EJ, Müller MR, Sommer RJ. 2013 Feeding plasticity in the nematode *Pristionchus pacificus* is influenced by sex and social context and is linked to developmental speed. *Evol. Dev.* **15**, 161–170. (doi:10.1111/ede.12030)
25. Bose N, Ogawa A, von Reuss SH, Yim JJ, Ragsdale EJ, Sommer RJ, Schroeder FC. 2012 Complex small-molecule architectures regulate phenotypic plasticity in a nematode. *Angew. Chem.* **51**, 12 438–12 443. (doi:10.1002/anie.201206797)
26. Maynard Smith J. 1982 *Evolution and the theory of games*. New York, NY: Cambridge University Press.
27. Sommer RJ, Carta LK, Kim SY, Sternberg PW. 1996 Morphological, genetic and molecular description of *Pristionchus pacificus* n. sp. (Nematoda: Neodiplogastridae). *Fund. Appl. Nematol.* **19**, 511–521.
28. Bongers T. 1999 The Maturity Index, the evolution of nematode life-history traits, adaptive radiation and cp-scaling. *Plant Soil* **212**, 13–22. (doi:10.1023/A:1004571900425)
29. Scholze VS, Sudhaus W. 2011 A pictorial key to current genus groups of 'Rhabditidae'. *J. Nematode Morphol. Syst.* **14**, 105–112.
30. Sudhaus W. 2011 Phylogenetic systemisation and catalogue of paraphyletic 'Rhabditidae' (Secernentea, Nematoda). *J. Nematode Morphol. Syst.* **14**, 113–178.
31. Choe A, von Reuss SH, Kogan D, Gasser RB, Platzer EG, Schroeder FC, Sternberg PW. 2012 Ascaroside signaling is widely conserved among nematodes. *Curr. Biol.* **22**, 772–780. (doi:10.1016/j.cub.2012.03.024)
32. Sulston J, Hodgkin J. 1988 Methods. In *The nematode* Caenorhabditis elegans (ed. WB Wood), pp. 587–606. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
33. Rae R, Iatsenko I, Witte H, Sommer RJ. 2010 A subset of naturally isolated *Bacillus* strains show extreme virulence to the free-living nematodes *Caenorhabditis elegans* and *Pristionchus pacificus*. *Environ. Microbiol.* **12**, 3007–3021. (doi:10.1111/j.1462-2920.2010.02278.x)
34. Iatsenko I, Boichenko I, Sommer RJ. 2013 *Bacillus thuringiensis* DB27 produces two novel toxins, Cry21Fa1 and Cry21Ha1, which act synergistically against nematodes. *Appl. Environ. Micrbiol.* **80**, 3266–3275. (doi:10.1128/AEM.00464-14)
35. Fürst von Lieven A. 2002 Functional morphology, origin and phylogenetic implications of the feeding mechanisms of *Tylopharynx foetida* (Nematoda: Diplogastrina). *Russ. J. Nematol.* **10**, 11–23.
36. Bumbarger D, Riebesell M, Rödelsperger C, Sommer RJ. 2013 System-wide rewiring underlies behavioral difference in predatory and bacterial-feeding nematodes. *Cell* **152**, 109–119. (doi:10.1016/j.cell.2012.12.013)
37. Angelo G, Van Gilst MR. 2009 Starvation protects germline stem cells and extends reproductive longevity in *C. elegans*. *Science* **326**, 954–958. (doi:10.1126/science.1178343)
38. Seidel HS, Kimble J. 2011 The oogenic germline starvation response in *C. elegans*. *PLoS ONE* **6**, e28074. (doi:10.1371/journal.pone.0028074)
39. Johnson TE, Mitchell DH, Kline S, Kemal R, Foy J. 1984 Arresting development arrests aging in the nematode *Caenorhabditis elegans*. *Mech. Ageing Dev.* **28**, 23–40. (doi:10.1016/0047-6374(84)90150-7)
40. Baugh LR, Sternberg PW. 2006 DAF-16/FOXO regulates transcription of *cki-1*/Cip/Kip and repression of lin-4 during *C. elegans* L1 arrest. *Curr. Biol.* **16**, 780–785. (doi:10.1016/j.cub.2006.03.021)
41. Pfennig DW. 1992 Polyphenism in spadefoot toad tadpoles as a locally adjusted evolutionary stable strategy. *Evolution* **46**, 1408–1420. (doi:10.2307/2409946)
42. DeWitt TJ, Sih A, Sloan Wilson D. 1998 Costs and limits of phenotypic plasticity. *Trends Ecol. Evol.* **13**, 77–81. (doi:10.1016/S0169-5347(97)01274-3)
43. Gilbert JJ. 1980 Further observations on developmental polymorphism and its evolution in the rotifer *Brachionus calyciflorus*. *Freshw. Biol.* **10**, 281–294. (doi:10.1111/j.1365-2427.1980.tb01202.x)
44. Denno RF, Olmstead KL, McCloud ES. 1989 Reproductive cost of flight capability: a comparison of life history traits in wing dimorphic planthoppers. *Ecol. Entomol.* **14**, 31–44. (doi:10.1111/j.1365-2311.1989.tb00751.x)
45. McCollum SA, Van Buskirk J. 1996 Costs and benefits of a predator-induced polyphenism in the gray treefrog *Hyla chrysoscelis*. *Evolution* **50**, 583–593. (doi:10.2307/2410833)
46. Miner BG. 2005 Evolution of feeding structure plasticity in marine invertebrate larvae: a possible trade-off between arm length and stomach size. *J. Exp. Mar. Biol. Ecol.* **315**, 117–125. (doi:10.1016/j.jembe.2004.09.011)
47. Ryals PE, Smith-Somerville HE, Buhse Jr HE. 2002 Phenotype switching in polymorphic *Tetrahymena*: a single-cell Jekyll and Hyde. *Int. Rev. Cytol.* **212**, 209–238. (doi:10.1016/S0074-7696(01)12006-1)
48. Kopp M, Tollrian R. 2003 Trophic size polyphenism in *Lembadion bullinum*: costs and benefits of an inducible offense. *Ecology* **84**, 641–651. (doi:10.1890/0012-9658(2003)084[0641:TSPILB]2.0.CO;2)
49. Gilbert JJ. 1973 Induction and ecological significance of gigantism in the rotifer *Asplanchia*

Downloaded from http://rspb.royalsocietypublishing.org/ on July 20, 2015

sieboldi. *Science* **181**, 63–66. (doi:10.1126/science.181.4094.63)

50. Collins JP, Cheek JE. 1983 Effect of food and density on development of typical and cannibalistic salamander larvae in *Ambystoma tigrinum nebulosum*. *Am. Zool.* **23**, 77–84.
51. Nyman S, Wilkinson RF, Hutcherson JE. 1993 Cannibalism and size relations in a cohort of larval ringed salamanders (*Ambystoma annulatum*). *J. Herpetol.* **27**, 78–84. (doi:10.2307/1564909)
52. Walls SC, Beatty JJ, Tissot BN, Hokit DG, Blaustein AR. 1993 Morphological variation and cannibalism in a larval salamander (*Ambystoma macrodactylum columbianum*). *Can. J. Zool.* **71**, 1543–1551. (doi:10.1139/z93-218)
53. Moczek AP. 2007 Developmental capacitance, genetic accommodation, and adaptive evolution. *Evol. Dev.* **9**, 299–305. (doi:10.1111/j.1525-142X.2007.00162.x)
54. Kanzaki N, Ragsdale EJ, Herrmann M, Sommer RJ. 2012 Two new species of *Pristionchus* (Rhabditida: Diplogastridae): *P. fissidentatus* n. sp. from Nepal and La Réunion Island and *P. elegans* n. sp. from Japan. *J. Nematol.* **44**, 80–91.
55. Ragsdale EJ, Kanzaki N, Röseler W, Herrmann M, Sommer RJ. 2013 Three new species of *Pristionchus* (Nematoda: Diplogastridae) show morphological divergence through evolutionary intermediates of a novel feeding polymorphism. *Zool. J. Linnean Soc.* **168**, 671–698. (doi:10.1111/zoj.12041)

Materials and Methods

(a) Nematode cultivation and phenotypes

Stock cultures of all nematode strains were kept on 6-cm plates with 1.7% nematode growth medium (NGM) agar and fed with a lawn of *Escherichia coli* OP50 grown in 400 µl L-Broth. Because the mouth dimorphism is a polyphenism in *P. pacificus*, both the Eu and St forms were available from a single isogenic culture, albeit with different frequencies as described below. Forms were selected for experiments by scoring their phenotypes as previously described [24]. Characters distinguishing the Eu form from the St form were the presence *vs.* absence of a subventral tooth, a claw-like *vs.* triangular dorsal tooth, and a wide *vs.* narrow mouth (Fig. 1). Phenotypes were determined using a Zeiss Discovery V.12 stereomicroscope, supplemented where necessary with differential interference contrast (DIC) microscopy on a Zeiss Axioskop.

All *P. pacificus* individuals subjected to functional and fitness assays were hermaphrodites. In experiments that introduced males as a source of sperm for assayed individuals, males were always well-fed young adults from bacterial culture. Assayed hermaphrodites were derived from multiple sibships, although inbred cultures of *P. pacificus*, which were perpetuated every generation from small numbers of hermaphrodites, ensured high homozygosity for each strain. Furthermore, the strain was maintained as a well-fed culture on bacteria for at least five generations prior to the experiment to control for any possible epigenetic effects. Therefore, assayed individuals were isolated from identical environmental and highly homozygous genetic backgrounds.

(b) Fecundity on a heterogeneous adult diet

As an alternative test for fecundity differences of the two forms on a prey diet, we measured the fecundity of mothers when offered bacteria following such a diet. Additionally, to test whether fecundity differences could be due to the availability of self-sperm, we offered a subset of prey-fed hermaphrodites both bacteria and mates. In the experiment, J4

hermaphrodites of *P. pacificus* RS5205 were fed for two days on prey as described above. After this feeding regimen, mothers of both mouth-forms were placed on individual NGM plates with (i) an excess (400 μl) of OP50, (ii) an excess of OP50 and one adult male of the same strain, or (iii) neither OP50 nor males. Nematodes were allowed to feed and, where possible, mate for three days, after which all living offspring were counted.

(c) Differential fecundity on a bacterial diet

Finally, to test whether differences in adult feeding-structures could impact lifetime fecundity on a strictly bacterial diet, we transferred pre-adult stage (J4) hermaphrodites of *P. pacificus* strain RS5205 individually to NGM plates seeded with an excess (400 μl) of OP50. The mouth phenotype of hermaphrodites was determined during the assay, upon nematodes reaching maturity. Hermaphrodites were allowed to lay eggs for two days and were transferred to new plates every two days until egg-laying ceased. Hatched larvae across all plates were counted. Additionally, we tested for differences of fecundity on a bacterium associated with *P. pacificus* in the wild, *Pseudomonas fluorescens* strain “Pento3” [22]. This assay was performed as on OP50, except prior to the assay nematode cultures were treated by bleaching to remove OP50 from stock culture plates and reared from eggs for one generation on *P. fluorescens*. The entire experiment was performed twice, each with sample sizes of n = 14-15 for each form on each food source. After confirming no significant differences between trials, results were pooled for a total sample size of n = 29-30 for each category.

(d) Statistical analyses

For the predation assay, proportions of successful Eu and St worms within each strain were compared using Pearson’s χ^2 test as implemented in R. Differences in predation measures (encounters per minute, attacks per encounter, and time required to kill) between Eu and St worms were carried out via Kruskal-Wallis ANOVAs, as Kolmogornov-Smirnov tests revealed departures from normality for most variables. To test for differences between the two forms in brood size on bacterial diets we performed a parametric test (Student’s *t*-test for independent samples), as distributions did not deviate

from normality. Kolmogornov-Smirnov, Kruskal-Wallis, and *t*-tests were conducted using Statistica v.9 (Statsoft). Kaplan-Maier survival curves were calculated and compared via log-rank tests, as implemented in Minitab 14. Differences in brood size (over specific days, and in total) between Eu and St worms, and between starved and prey-fed worms, were examined using Kruskal-Wallis ANOVAs as described above.

Figure S1. Predatory behaviour of *Pristionchus pacificus* JU482, as measured by encounters of prey per minute and attacks per prey individual encountered. Box plots show the median (centre line), the lower and upper quartiles (box bounds), and the range limits (whiskers). (*a*) Encounters per minute. For both successful and non-successful acts of predation, Eu individuals did not encounter prey more often than St individuals. (*b*) Attacks per encounter. Neither mouth-form showed more attacks for a given number of opportunities to make them.

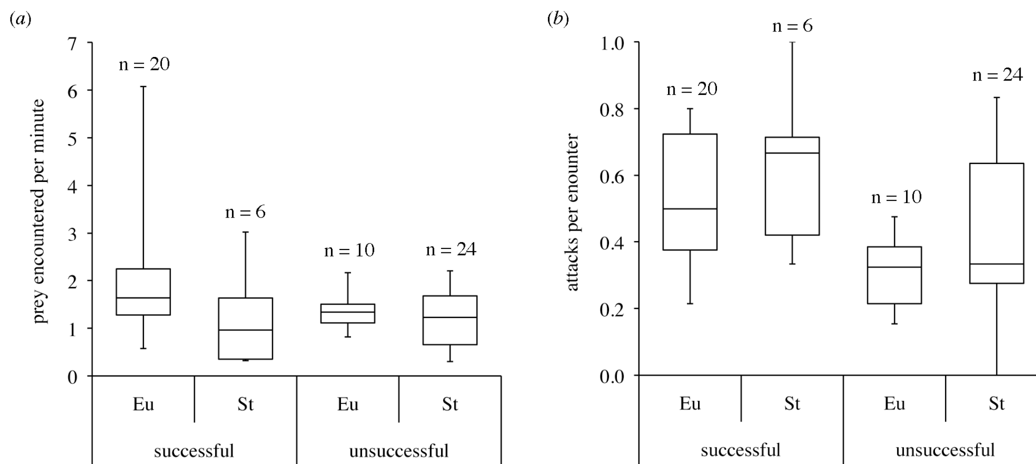
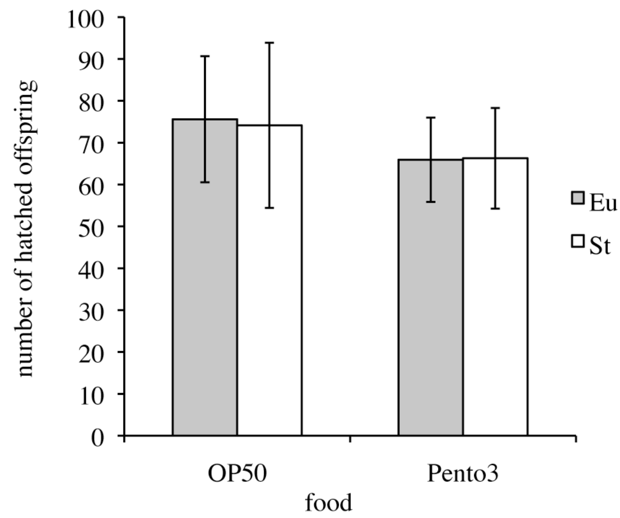


Figure S2. Fecundity (brood size) when fed *ad libitum* on bacteria. Food sources were *Escherichia coli* OP50 and the naturally associated strain *Pseudomonas fluorescens* “Pento3.” No differences were detected between eurystomatous (Eu) and stenostomatous (St) individuals, indicating no fecundity advantage for either form in the presence of abundant bacterial food.



Chromatin remodeling and antisense-mediated up-regulation of the developmental switch gene *eud-1* control a predatory feeding decision

Vahan Serobyan[1], Hua Xiao[1,2], Christian Rödelsperger[1], Suryesh Namdeo[1], Waltraud Röseler[1], Hanh Witte[1] & Ralf J. Sommer[1,3]

[1]Department for Evolutionary Biology, Max-Planck-Institute for Developmental Biology, Spemannstrasse 35, 72076 Tübingen, Germany

[2]Current address: University of Missouri-Columbia, Division of Biological Sciences 103 Tucker Hall, Columbia, MO 65211 USA

[3]Correspondence: ralf.sommer@tuebingen.mpg.de (R.J.S.)

Running title: Chromatin remodeling regulates predatory feeding

Abstract

Developmental plasticity has been suggested to facilitate morphological novelty and diversity through developmental switches[1-5], but little is known about the molecular aspects of developmental switch mechanisms underlying plasticity. We have recently identified the sulfatase *eud-1* as part of a developmental switch controlling morphological plasticity that governs a predatory *vs.* bacteriovorous mouth dimorphism in *Pristionchus* nematodes[6]. EUD-1 regulates the execution of one of two alternative mouth-forms and was shown to have a key role in micro – and macroevolutionary divergence. However, the molecular mechanisms underlying *eud-1* regulation are currently unknown. Here, we show that mutations in the conserved histone acetyltransferase *Ppa-lsy-12* and the methyl-binding-protein *Ppa-mbd-2* mimic the *eud-1* phenotype, resulting in the absence of one of the mouth-forms. Mutations in both genes cause histone modification defects and reduced *eud-1* expression. Surprisingly, *Ppa-lsy-12* mutants also result in the down-regulation of an antisense-*eud-1* RNA, and further experiments suggest that antisense-*eud-1* acts through *eud-1* itself. Indeed, overexpression of the antisense-*eud-1* RNA from transgenes increases the *eud-1*-sensitive mouth-form in wild-type animals and results in increased *eud-1* expression. In contrast, this effect is absent in *eud-1* mutants indicating that antisense-*eud-1* positively regulates *eud-1*. Our experiments identify chromatin remodeling and antisense-mediated up-regulation as integral parts of developmental switch mechanisms controlling phenotypic plasticity. These epigenetic mechanisms provide a theoretical framework for linking genetic regulation to environmental input.

Keywords: *Pristionchus pacificus*, developmental plasticity, predatory feeding, developmental switch mechanisms, sulfatase

Main text

P. pacificus and related nematodes live in a necromenic association with scarab beetles, a life style facilitated by dynamic feeding mode switching between bacterial grazing and the predation of other nematodes (Fig. 1a,b)[7]. This feeding diversity relies on the presence of moveable teeth and *Pristionchus* nematodes exhibit two distinct morphs – stenostomatous (St, narrow-mouthed) or eurystomatous (Eu, wide-mouthed) – that differ in the number and shape of associated teeth and the size and form of the buccal cavity[8] (Fig. 1c,d). When fed on *E. coli* OP50 bacteria under lab conditions, *P. pacificus* California reference strain RS2333 hermaphrodites have a stable 70%:30% Eu:St ratio, but this can be influenced by starvation, crowding and pheromone signaling[8-10]. Because *P. pacificus* hermaphrodites reproduce primarily by selfing, strains are genetically homogeneous, and the presence of two distinct morphs thus represents an example of developmental plasticity, which was also demonstrated experimentally[8].

The existence of developmental switch mechanisms is essential for the irreversible control of plasticity, but associated mechanisms are largely unknown. We have recently identified the sulfatase *eud-1* as part of a genetic network that constitutes the developmental switch for the *P. pacificus* mouth-form decision[6]. In *eud-1* mutants the Eu form is absent (*eud*, *eu*rystomatous-form-*d*efective), whereas overexpression from transgenes fixes the Eu form, thus confirming that EUD-1 acts as a developmental switch[6]. *eud-1* is X-linked and dosage-dependent, and it regulates differences in mouth-form frequency between hermaphrodites and males, among *P. pacificus* strains, and between *Pristionchus* species[6]. Interestingly, *P. pacificus eud-1* derives from a recent duplication that resulted in two neighboring gene copies arranged in a head-to-head orientation (Fig. 1e). These paralogs are highly similar at the sequence level, but only *eud-1* is expressed in a small number of *P. pacificus* head neurons, where its expression is sufficient to induce the execution of the Eu mouth-form[6]. However, while *eud-1* expression is highly regulated, the underlying mechanisms that control this developmental switch gene remain unknown.

To study the regulation of *eud-1,* we searched for pleiotropic mutants

with a Eud phenotype in hermaphrodites similar to *eud-1*. By screening more than 30 already-established mutant strains with egg laying-, vulva-, or sex determination defects, we identified two mutants, *tu319* and *tu365,* with a nearly complete loss of the Eu form (Fig. 2a). While *tu319* was previously molecularly uncharacterized, *tu365* represents a deletion allele in the methyl-binding protein family member *Ppa-mbd-2* (ref. 11). *Ppa-mbd-2(tu365)* is recessive, homozygous viable, and displays both a fully penetrant egg-laying defect and a complete absence of the Eu mouth-form (Fig. 2a). *Ppa-mbd-2(tu365)* contains a 1.7 kb deletion that removes four of six exons, suggesting that the absence of the Eu form results from a strong reduction-of-function or even null mutation in this gene. Thus, the phenotype of *mbd-2* in *P. pacificus* reveals the existence of pleiotropic regulators of mouth-form plasticity.

In *tu319* mutants, only 2% of hermaphrodites have a Eu mouth-form (Fig 2a). *tu319* was isolated in a screen for vulva-defective mutants and represents one of three alleles of the previously genetically characterized *vul-2* (*vul*valess) locus[12]. Interestingly, only *tu319* but not the two other alleles, *tu18* and *tu30,* show mouth-form defects indicating that the effect of *vul-2* on mouth-form regulation is allele-specific. We mapped *tu319* to the tip of chromosome IV in proximity to the marker S210 (SFig. 1a). Sequencing of fosmid clones of this gene poor region resulted in the identification of a histone-acetyltransferase orthologous to the *Caenorhabditis elegans* gene *lsy-12* (SFig. 1b,c)[13]. Sequencing of *lsy-12* identified mutations in all three alleles; *tu319* and *tu30* have splice-site mutations, whereas *tu18* contains a 598bp deletion in the putative 3´ end of the gene (SFig. 1b). *Ppa-lsy-12* is a complex gene with more than 30 predicted exons, and RACE and RNA seq experiments provide strong evidence for extensive alternative splicing (SFig. 1b). *Ppa-lsy-12* has a typical MYST domain in the 5´ part of the gene encoded by exons 5-13 (SFig. 1b), which is present in the majority of transcripts. Interestingly, *tu319* affects the splice site of exon 7, whereas the two other alleles affect the 3´ part of the gene, which is not associated with known protein domains and is not present in the majority of transcripts.

To attempt phenotypic rescue, we generated a construct of *Ppa-lsy-12* containing exons 1-20 (see Methods) and obtained three independently

transformed lines carrying the *Ppa-lsy-12* construct alongside an *egl-20::rfp* (red fluorescent protein) reporter. All three transgenic lines rescued both, the vulvaless defect and the mouth-form defect of *tu319* (SFig. 1c,d). Specifically, in transgenic animals the mouth-form was 71% Eu (versus 2% Eu in *tu319* worms) and in one line studied in greater detail 90% of the vulva precursor cells were induced to form vulval tissue (versus 33% in *tu319* worms). These results indicate that *vul-2* is indeed identical to *Ppa-lsy-12* and we renamed the gene accordingly. Taken together, two evolutionarily conserved genes, *Ppa-lsy-12* and *Ppa-mbd-2*, are pleiotropic regulators of mouth-form plasticity and mutations in these genes result in a strong reduction or absence of the Eu mouth-form.

The molecular nature of *Ppa-lsy-12* suggests that chromatin remodeling may control the developmental switch mechanism that underlies the *P. pacificus* mouth dimorphism. Chromatin remodeling proteins regulate numerous developmental processes[14], but nothing is known of a potential role for chromatin remodeling in the regulation of developmental plasticity. Therefore, we first asked if histone modifications are indeed altered in *lsy-12* and *mbd-2* mutants. We isolated proteins from mixed stage cultures of wild type, *mbd-2,* and *lsy-12* mutant animals and found changes of four histone marks using antibody staining (Fig. 2b). H3K4me3 is strongly reduced in both *mbd-2* and *lsy-12* mutant animals, whereas H3K4me2 is reduced only in *mbd-2* mutants (Fig. 2b). In contrast, H3K4me1 and several other histone marks are not affected (SFig. 2a). In addition to H3K4 methylation, the acetylation of H3K27 is strongly and that of H3K9 moderately reduced in both mutants (Fig. 2b). These findings demonstrate a role for MBD-2 and LSY-12 in chromatin remodeling in *P. pacificus.* Furthermore, because H3K4 methylation and acetylation of various H3 lysines are often found as gene activation marks[14], these results suggest that the effects of *mbd-2* and *lsy-12* on mouth-form developmental plasticity is a consequence of chromatin remodeling-mediated transcriptional regulation.

Next, we tested the developmental switch gene *eud-1* as a candidate target of chromatin remodeling by LSY-12 and MBD-2. First, we studied *eud-1* expression by performing qRT-PCR experiments in wild type and mutant hermaphrodites in different developmental stages. Indeed, *eud-1* is significantly down regulated in *mbd-2* and *lsy-12* mutants, in J2 worms, the larval stage at

which the mouth-form is determined (Fig 2c). In addition, we also observed *eud-1* down-regulation in adult stages, suggesting that *eud-1* expression is similarly controlled throughout development (SFig. 2b). These results suggest that the mouth-form defects of *mbd-2* and *lsy-12* mutants results from down-regulation of *eud-1*. Interestingly, these effects of *mbd-2* and *lsy-12* mutants on *eud-1* expression levels and the mouth-form frequency qualitatively match the patterns seen in *P. pacificus* males and highly-St wild isolates[6].

To further explore the function of chromatin remodeling on the regulatory network controlling the developmental switch, we used RNAseq to compare the transcriptomes of wild type and *Ppa-lsy-12* mutant animals (Fig. 3a). Consistent with our RT-PCR results, *eud-1* expression was heavily down regulated in *Ppa-lsy-12* worms ($P<10^{-8}$). Surprisingly however, we also found a strong effect on previously uncharacterized antisense reads of *eud-1* (Fig. 3a). Indeed, additional RT-PCR experiments identified an antisense *eud-1* transcript, termed as-*eud-1*. The as-*eud-1* RNA consists of three exons with a total size of 863 nucleotides, some of which is antisense to *eud-1* coding exons, including *eud-1* exons 7-10 and exon 19 (Fig. 3a). as-*eud-1*has no open reading frame suggesting that it encodes a long non-coding (lnc) RNA (SFig. 3).

To study the functional significance of this lnc RNA for the mouth-form decision, we generated transgenic animals in which the as-*eud-1* cDNA is placed under the *eud-1* promoter. We generated transgenic animals in a wild type background in order to be able to score the potential effects of as-*eud-1*. We obtained three independent transgenic lines, all of which showed a strong masculinization phenotype resulting in more than 95% of animals being males. These transgenic lines showed no embryonic lethality and transgenic males were successfully mated indicating that the high incidence of males result from as-*eud-1*-induced X chromosome–specific non-disjunction, a phenomenon known from various *C. elegans* mutants such as *him-8* (ref. 15). We therefore used the male mouth-form frequency to study the influence of as-*eud-1*. In contrast to hermaphrodites, spontaneous wild type males are only 10-20% Eu because *eud-1* is X-linked and dosage-sensitive (Fig. 1e)[6,10]. The male mouth-form phenotype should be shifted towards more St animals in case of a negative effect and towards higher Eu frequencies in case of a positive function of the as-*eud-1*RNA.

We made the remarkable finding that as-*eud-1* has a positive function on the Eu *vs.* St mouth-form decision and *eud-1* expression (Fig. 3b,c), whereas most cases of antisense-mediated regulation results in transcriptional down regulation[16]. Three experiments result in this finding. First, the frequency of the *eud-1*-sensitive mouth-form was dramatically increased in transgenic lines carrying as-*eud-1* in a wild type background (from 20% to 64% Eu males) (Fig. 3b). Second, qRT-PCR experiments revealed a strong up-regulation of *eud-1* in as-*eud-1* transgenic males (Fig. 3c). Third, as-*eud-1* transgenes in a *eud-1* mutant background also caused a high incidence of males, but without affecting male mouth-form. Specifically, *eud-1(tu445)*;Ex(as-*eud-1*) males were completely St (Fig. 3b), indicating that as-*eud-1* acts through *eud-1*. Taken together, these experiments suggest that chromatin remodeling acts through antisense-mediated up-regulation of *eud-1*.

as-*eud-1* is expressed at very low copy numbers that can most reliably be detected by RNAseq (Fig. 3a). Also, nested RT-PCR experiments with as-*eud-1*-specific primers reliably amplified the full-length lnc RNA, whereas qRT-PCR experiments failed to reveal transcripts above background level, a phenomenon known from other lnc RNAs[17]. Similarly, fluorescent *in-situ* hybridization (FISH) experiments were able to detect *eud-1* in several head neurons (SFig. 4) as previously reported[6], but as-*eud-1* transcripts were undetectable. The intertwined nature of the *eud-1* and as-*eud-1* RNAs makes it unfeasible to create mutations that only affect as-*eud-1* without interfering with *eud-1* coding or potential regulatory sequences. Therefore, as-*eud-1* function can currently not be studied genetically without interfering with *eud-1* itself and it remains unknown if as-*eud-1* acts in *cis* or *trans*. However, sequence comparison of the *P. pacificus* *eud-1* locus (SFig. 5) with that of other *Pristionchus* species suggests a conserved role of as-*eud-1* in *eud-1* regulation.

Developmental switching represents an appealing concept to link genetic and environmental influences on phenotypically plastic traits. Our studies of the sulfatase *eud-1* – its function as a developmental switch, its role in micro- and macroevolutionary divergence and, here its regulation – provide such mechanistic insights. Previous characterization of *eud-1* resulted in several surprising findings, i.e. its recent origin by gene duplication and its epistasis to

other factors controlling feeding plasticity, thus demonstrating the importance of terminal addition of new genes for regulating plasticity[6]. We have now shown that two evolutionarily conserved genes, *mbd-2* and *lsy-12*, are involved in the transcriptional regulation of *eud-1* providing first insight into the molecular mechanisms underlying the regulation of developmental switches. In particular, the involvement of antisense-mediated up-regulation of *eud-1* indicates an unexpected complexity and results in three major conclusions. First, our findings demonstrate that the role of *eud-1* involves complex regulation of its own transcription. We previously observed that the coding region of *eud-1* is subject to strong purifying selection, and our new findings support and extend these conclusions regarding the importance of regulatory *vs.* structural changes. Second, we demonstrate the involvement of chromatin remodeling in the developmental switch mechanism regulating mouth-form plasticity in *P. pacificus*. We speculate that chromatin remodeling represents a powerful epigenetic mechanism that might link environmental signals to transcriptional regulation of plasticity. Third, we provide evidence for an antisense RNA in up-regulation. Transcriptional surveys of many eukaryotes have uncovered hundreds of noncoding transcripts[18] and though many of these function as transcriptional regulators, most do so as inhibitors. Conversely, antisense-mediated transcriptional activation or maintenance has only rarely been described[17,19]. Thus, the example of as-*eud-1* regulation of *eud-1* reveals complex regulatory mechanisms that can serve as model to link genetic and environmental control of developmental plasticity in future studies.

Methods Summary

All wild-type worms were *P. pacificus* reference strain RS2333. In all experiments phenotypes were scored using a Zeiss Discovery V12 stereomicroscope and the identification of mouth-forms relied on several morphological markers as described previously[6,8,10]. Transgenic animals were generated as described by Schlager et al (2009) (ref. 20). For phenotypic rescue of *vul-2*, the germ line of *vul-2(tu319)* mutant animals was injected with a 17 kb

genomic construct containing exons 1-20 of *Ppa-lsy-12* and 4.5 kb of flanking regulatory region (2ng/µl), the marker *Ppa-egl-20*::*TurboRFP* (10ng/µl) and genomic carrier DNA (60ng/µl) from the recipient strain. To study the as-*eud-1* lnc RNA, we generated a 7.5 kb construct consisting of approximately 6.5 kb promoter element and the 863 bp cDNA fragment of as-*eud-1*. This construct was injected (2ng/µl) with the *Ppa-egl-20*::*TurboRFP* (10ng/µl) marker and genomic carrier DNA (60ng/µl) of *P. pacificus* RS2333 and *Ppa-eud-1(tu445)*, respectively. For all constructs, at least two independent transgenic lines were generated and transgenic animals were scored over multiple generations. Transgenic lines containing the as-*eud-1* lnc RNA yielded more than 95% male progeny and all lines were kept at least until the tenth generation. No embryonic lethality was observed in association with these transgenes. Transgenic males were crossed with *Ppa-pdl-1* (ref. 21) and wild-type hermaphrodites and cross-progeny was readily observed.

Full Methods and any associated references are available in the Supplementary Material.

Acknowledgments

We thank Drs. C. Weadick, J. Lightfoot and A. Streit for comments on the manuscript and members of the Sommer lab for discussion.

Author contributions

V.S. performed mouth-form and transgenic experiments, histone modification assays and RT-PCR experiments. H.X. physically cloned *vul-2*, S.N. performed FISH experiments and C.R. performed all computational studies. W.R. and H.W. were involved in the molecular parts of the project and R.J.S. led the overall project and wrote the manuscript with assistance from S.V. and C.R.

Figure Legends

Fig. 1 Developmental plasticity in *P. pacificus* and its regulation by the developmental switch gene *eud-1*. a, The Oriental beetle *Exomala orientalis* is one of the beetle hosts with which *P. pacificus* lives in a necromenic association. **b,** Scanning electron micrograph showing *P. pacificus* predatory feeding on a small larva of *C. elegans* (white arrow). **c, d,** Mouth dimorphism of *P. pacificus* enabling a switch between bacterial grazing and predatory feeding. Stenostomatous (St) animals (c) have a narrow buccal cavity (light blue arrow) and a flint-like dorsal tooth (red arrow), but miss the subventral tooth. In contrast, eurystomatous (Eu) animals (d) have a wide buccal cavity (light blue arrow), a claw-like dorsal tooth (red arrow) and an additional subventral tooth (blue arrow). **e,** Molecular organization of the *eud-1* locus and effect of *eud-1* function on mouth-form ratios. *eud-1* derives from a recent gene duplication, with the neighboring sulfatase *sul-2.2.1* arranged in a head-to-head orientation. The two genes are separated by a 7.5 kb intergenic region that when used as promoter drives the expression of *eud-1* in various head neurons. In wild-type animals, hermaphrodites and males form approximately 70% and 10% Eu animals, respectively. In *eud-1* mutants, both sexes are completely St, whereas *eud-1* overexpression causes both genders to form only Eu animals indicating that EUD-1 functions as developmental switch.

Fig. 2 Mouth-form defects of two pleiotropic mutants and their effect on histone modification and *eud-1* expression. a, *Ppa-lsy-12(tu319)* and *Ppa-mbd-2(tu365)* result in the (nearly) complete absence of Eu hermaphrodites, similar to *eud-1* mutants. Data are presented as the total Eu frequency, n>100 for all strains. **b,** *Ppa-lsy-12* and *Ppa-mbd-2* mutants result in severe histone modification defects. **c** qRT-PCR experiments reveal down regulation of *eud-1* expression in *Ppa-lsy-12* and *Ppa-mbd-2* mutants relative to wild type in J2 larvae. *p<0.05 and ***$p<10^{-5}$, Kruskal-Wallis test.

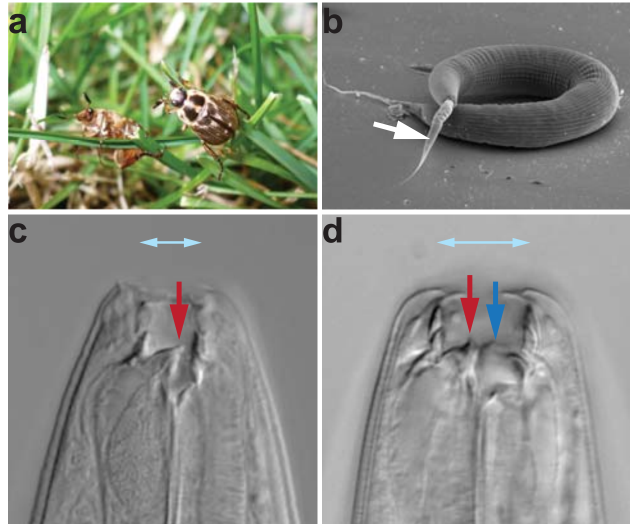
Fig. 3 Molecular organization and function of as-*eud-1*. a, Organization of the *eud-1* and antisense *eud-1* (as-*eud-1*) locus and RNAseq experiments of wild type

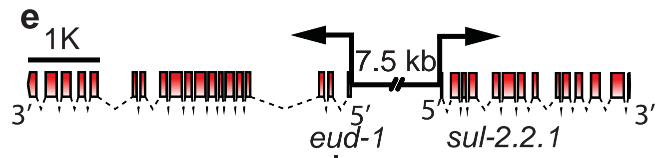
and *Ppa-lsy-12* mutant animals. The long noncoding RNA as-*eud-1* consists of three exons that span large parts of the *eud-1* coding region. The structure of as-*eud-1* was identified in RT-PCR experiments and revealed the existence of a short exon, which went undetected in RNAseq. Other antisense reads obtained at lower frequency in the RNAseq experiment, were not confirmed to be part of as-*eud-1* in RT-PCR experiments with mixed stage wild type animals. Subsequent panels show sense and antisense expression as measured for wild type (wt) and *Ppa-lsy-12* mutant animals. Note that nearly no reads of *eud-1* and as-*eud-1* were observed in *Ppa-lsy-12* mutants. **b,** Transformation of wild type hermaphrodites with as-*eud-1* cDNA induced a high incidence of males and a Eud phenotype in male progeny. In contrast, transformation of *eud-1(tu445)* mutant animals with as-*eud-1* did not result in a Eud phenotype, although the high incidence of males was similar to the transformation of wild type animals. **c,** qRT-PCR experiments reveal an up-regulation of *eud-1* in as-*eud-1* transgenic males relative to wild type males. *$p<0.05$ and ***$p<10^{-5}$, Kruskal-Wallis test.

References:

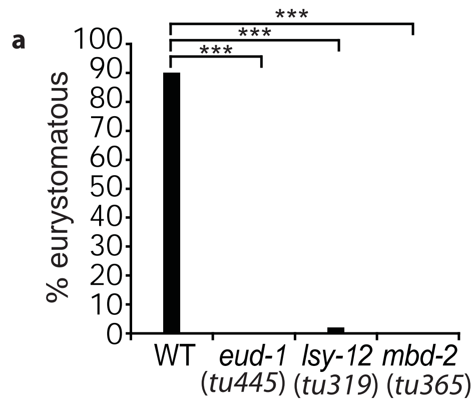
1. West-Eberhard, M.-J. *Developmental plasticity and evolution.* Oxford University Press, Oxford. (2003).
2. Moczek, A.P., Sultan, S., Foster, S., Ledón-Rettig, C., Dworkin, I., Nijhout, F., Abouheif, E., and Pfennig, D.W. The role of developmental plasticity in evolutionary innovation. *Proc. R. Soc. B 278*, 2705-2713 (2011).
3. Nijhout, H. F. *Insect hormones.* Princeton University Press, Princeton (2003).
4. Schlichting, C.D. Origins of differentiation via phenotypic plasticity. *Evol. Dev. 5*, 98-105 (2003).
5. Pigliucci, M. *Phenotypic plasticity: Beyond nature and nurture*. John Hopkins University, Baltimore (2001).
6. Ragsdale, E., Müller, M., Roedelsperger, C. & Sommer, R. J. A developmental switch coupled to the evolution of plasticity acts through a sulfatase*. Cell,* **155**, 922-933 *(*2013).
7. Herrmann, M., Mayer, W., Hong, R., Kienle, S., Minasaki, R. & Sommer, R. J. The nematode *Pristionchus pacificus* (Nematoda: Diplogastridae) is associated with the Oriental beetle *Exomala orientalis* (Coleoptera: Scarabaeidae) in Japan. *Zool. Science*, **24**, 883-889 (2007).
8. Bento, G., Ogawa, A. & Sommer, R. J. Co-option of the endocrine signaling module Dafachronic Acid-DAF-12 in nematode evolution. *Nature*, **466**, 494-497 (2010).
9. Bose, N., Ogawa, A., von Reuss, S. H., Yim, J. J., Ragsdale, E. J., Sommer, R. J. & Schroder, F. C. Complex small molecular architectures regulate phenotypic plasticity in a nematode. *Angew. Chemie*, 51, 12438-12443 (2012).
10. Serobyan, V., Ragsdale, E., Müller, M. & Sommer, R. J. Feeding plasticity in the nematode *Pristionchus pacificus* is influenced by gender and social context and is linked to developmental speed. *Evolution & Development*, **15**, 173-182 (2013).
11. Gutierrez, A. & Sommer, R. J. Functional diversification of the *mbd-2* gene between *Pristionchus pacificus* and *Caenorhabditis elegans*. BMC Genetics, **8**, 57 (2007).
12. Sigrist, C. & Sommer, R.J. Vulva formation in *Pristionchus pacificus* relies on continuous gonadal induction. *Devel., Gen. Evol.* **209**, 451-459 (1999).
13. Sarin, S., Bertrand, V., Bigelow, H., Boyanov, A., Doitsidou, M., Poole, R., Narula, S., & Hobert, O. Analysis of multiple ethyl methanesulfonate-mutagenized *Caenorhabditis elegans* strains by whole-genome sequencing. *Genetics,* **185**, 417-30 (2010).
14. Musselman, C. A., Lalonde, M.-E.Cote, J. & Kutateladze T. G. Perceiving the epigenetic landscape through histone readers. *Nat. Struct. Mol. Biol.* **19**, 1218-1227 (2012).
15. Hodgkin, J.A., Horvitz, H. R. & Brenner, S. Nondisjunction mutants on the nematode *Caenorhabditis elegans*. Genetics 91, 67-94 (1979).
16. Rinn, J. L. & Chang, H. Y. Genome regulation by long noncoding RNAs. *Annu. Rev. Biochem*. **81**, 145-166 (2012).

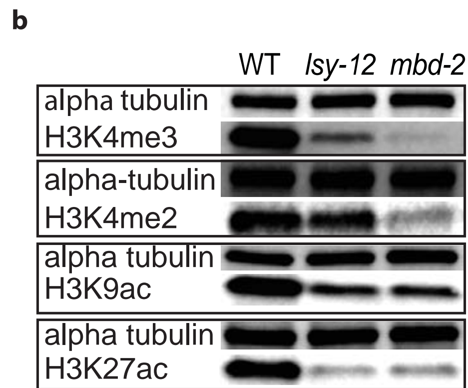
17. Dimitrova, N. et al., *LincRNA-p21* activates *p21* in *cis* to promote polycompb target gene expression and to enforce the G1/S checkpoint. Mol. Cell 54, 1-14 (2014).
18. Guttman, M. et al., Chromatin signature reveals over a thousand highly conserved large non-coding RNAs in mammals*. Nature* **458**, 223-227 (2009).
19. Sakurai, I., Stazic, D., Eisenhut, M., Vuorio, E., Steglich, C., Hess, W. R. & Aro, E.-M. Positive regulation of *psbA* gene expression by cis-encoded antisense RNAs in *Synechocystis* sp. PCC6803. *Plant Physiology* **160**, 1000-1010 (2012).
20. Schlager, B., Wang, X., Braach, G. & Sommer, R. J. Molecular cloning of a dominant Roller mutant and establishment of DNA-mediated transformation in the nematode model *Pristionchus pacificus*, *Genesis*, **47**, 300-304 (2009).
21. Witte, H., Moreno, E., Rödelsperger, C., Kim, J., Kim, J.-S., Streit A. & Sommer, R. J. Gene inactivation using the CRISPR/Cas9 system in the nematode *Pristionchus pacificus, Dev Genes & Evol.*, **225**, 55-62 (2015).

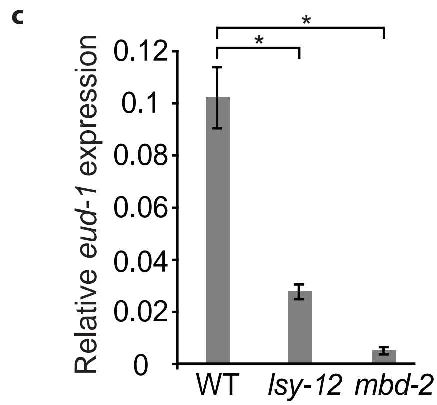




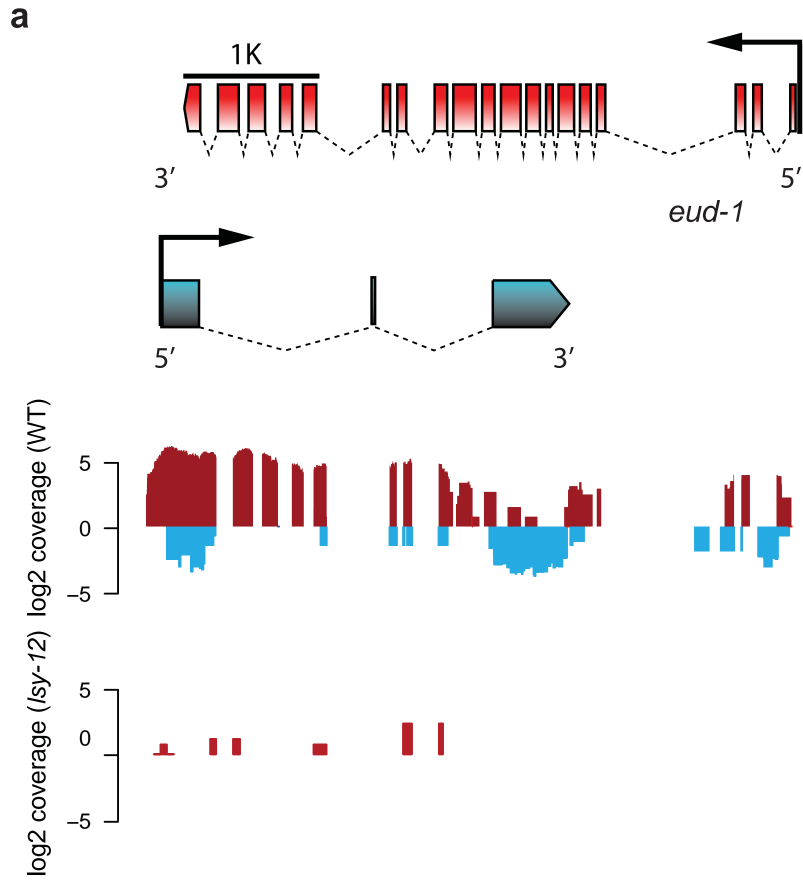
		Eu	St
WT	⚥	70%	30%
	♂	10%	90%
eud-1	⚥	0%	100%
	♂	0%	100%
Ex[*eud-1*^{CA}]	⚥	100%	0%
	♂	100%	0%


a
***
***
***
100
90
80
70
60
50
40
30
20
10
0
% eurystomatous
WT
eud-1 (tu445)
lsy-12 (tu319)
mbd-2 (tu365)

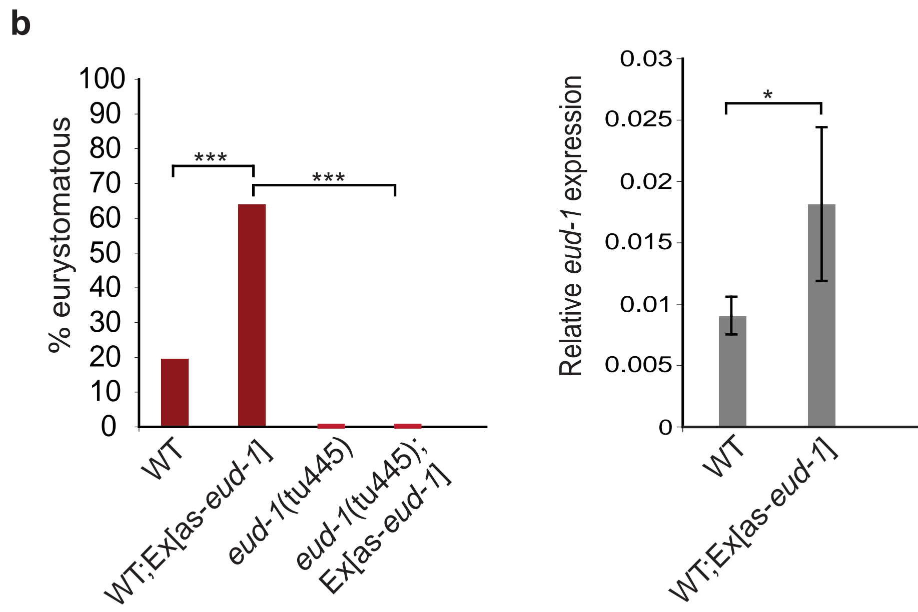

b
WT
lsy-12
mbd-2
alpha tubulin
H3K4me3
alpha-tubulin
H3K4me2
alpha tubulin
H3K9ac
alpha tubulin
H3K27ac


c
*
*
0.12
0.1
0.08
0.06
0.04
0.02
0
Relative eud-1 expression
WT
lsy-12
mbd-2

a



b



Supplementary Material

Chromatin remodeling and antisense-mediated up-regulation of the developmental switch gene *eud-1* control a predatory feeding decision

Vahan Serobyan, Hua Xiao[#], Christian Rödelsperger, Suryesh Namdeo, Waltraud Röseler, Hanh Witte & Ralf J. Sommer[*]

Content

Methods 2
Stables 7
Sfigures and legends 9

METHODS

Culture Conditions

All *Pristionchus* strains were kept on 6-cm plates with nematode growth medium (NGM) agar and were fed with a lawn of *Escherichia coli* OP50 grown in 400 µl L-Broth. Cultures were maintained at 20ºC. Because the mouth-form ratio is sensitive to unknown environmental factors[6], all experiments include their own controls for the wild-type Eu frequency. Also, to minimize the potential for laboratory evolution of the trait, a new culture of the California (RS2333) strain was revived annually from a frozen voucher.

Phenotype Scoring

The mouth-form phenotype was scored as previously described[10]. In short, characters used to discriminate between Eu and St individuals, respectively, were (i) the presence *vs.* absence of a subventral tooth, (ii) a claw-like *vs.* flint-like or triangular dorsal tooth, and (iii) a wide *vs.* narrow stoma (mouth). Characters (i) and (ii) were discrete, non-overlapping, and sufficient to distinguish the two forms. Apparent intermediates between the two forms were rare (<0.1%) and were not included in counts. Phenotypes could be scored using Zeiss Discovery V.12 stereomicroscopes and were supplemented where necessary with differential interference contrast (DIC) microscopy on a Zeiss Axioskop.

Mapping of *vul-2(tu319)* and Mutant Identification

For genetic mapping, mutants in the California background were crossed to the Washington strain (PS1843). F2 progeny were cloned and screened for two generations to confirm the mutant phenotype and the homozygosity of mutations. Genomic DNA of outcrossed mutant lines was extracted for genetic mapping. Simple-sequence conformation (SSCP) polymorphism markers were tested against 30-40 outcrossed mutant lines and detected as previously described[22,23]. *vul-2* was mapped to the tip of chromosome IV close to the marker S210. Further mapping localized *vul-2* to the bacterial-artificial-chromosome (BAC) clone BACPP16-M16 and the fosmid subclones 525-J06, 543-P16 and 558-O23. Light shotgun sequencing of these fosmid clones

resulted in the identification of *Ppa-lsy-12* as candidate gene for *vul-2*. To prepare samples for whole-genomic sequencing, DNA was extracted from all three alleles *tu18*, *tu30* and *tu319* and mutations were identified in all three alleles.

Alternative splicing of *Ppa-lsy-12*

Following preparation of mixed-stage RNA libraries for *P. pacificus* RS2333, coding DNA (cDNA) was amplified by reverse transcriptase PCR and sequenced. 5' and 3' RACE experiments were performed by SMARTer RACE cDNA Amplification Kit following the manufacturer's protocol (Life Technologies). The full list of gene-specific primers that were designed according to the available genomic sequence for *Ppa-lsy-12* is provided in Stable 1.

RNA-Sequencing Experiments

Presence and levels of gene expression were measured by whole-transcriptome sequencing (RNA-Seq) of *Ppa-lsy-12(tu319)* mutants and *P. pacificus* wild types. Culture populations were allowed to grow until their food was exhausted, immediately after which the cultures were processed for sequencing. Five mixed-stage plates were washed with 40 ml M9, centrifuged immediately at 1300 g for 4 min, rinsed with 40 ml 0.9% NaCl treated with 40 µl ampicillin and 40 µl chloramphenicol and shaken gently for 2 hours, and finally concentrated into a pellet by centrifugation and immediately frozen in liquid nitrogen. NEBNext Ultradirectional RNA Library Kit (Cat # E7420L) was used to prepare libraries. RNA-Seq libraries were sequenced as 2x 100-bp paired-end reads on an Illumina HiSeq 2000, yielding 11-45 million paired-end reads per sample. Raw reads were aligned to the reference genomes of *P. pacificus* (Hybrid1) (www.pristionchus.org), using the software Tophat v.2.0.3 (ref. 24). Expression levels were estimated and compared using the programs Cufflinks and Cuffdiff v.2.0.1 (ref. 24).

qRT-PCR

Total RNA from synchronized cultures was isolated using TRIzol (ambion by life technologies). For reverse transcription Superscript II reverse

transcriptase (Invitrogen, Cat.No: 18064) was used following the manufacturer's instructions. We used 1μg total RNA. The qRT-PCR experiments were performed on a LightCycler 480 system; using SybrGreen (Roche Diagnostics) with a reaction set up described elsewhere[26]. To detect *eud-1* expression we used VSe13F GATGATCGAGTCACACAGATCCG forward and VSe13R ATGTAGTAGGAGAGTTGAGCAGCG reverse primers. *Ppa-ama-1*, *Ppa-cdc-42* and *Ppa-Y45F10D.4* were used as reference genes as previously described[26]. PCR efficiencies were determined using external standards on plasmid mini-preparation of cloned PCR-products. Expression levels were analyzed by basic relative quantification. We performed 2-6 biological replicates for different experiments.

as-*eud-1* transcript analysis

RNAseq reads of wild type worms cover the majority of *eud-1* exons to a similar extend. In addition, we observed antisense reads at the *eud-1* locus that were previously uncharacterized. These antisense reads are expressed at very low levels and cannot be detected in qRT-PCR experiments, which otherwise are used as a standard procedure in *P. pacificus* (see above). We used many different PCR primer combinations (Stable 3) in a variety of nested PCR experiments to study which of the antisense reads if any are present in a potential as-*eud-1* cDNA. These experiments revealed the existence of one transcript of 863 nucleotides that consists of three exons (Fig. 3, SFig. 5). The two larger exons cover exactly those reads that were most abundantly found in the RNAseq experiment of wild type worms. However, exon 2 consists of only 26 nucleotides and went unnoticed at the RNAseq level,

Genetic Transformation

Transgenic animals were generated as described by Schlager and co-workers[20]. For phenotypic rescue of *vul-2*, the germ line of *vul-2(tu319)* mutant animals was injected with a 17 kb genomic construct containing exons 1-20 of *Ppa-lsy-12* and 4.5 kb of flanking regulatory region (2ng/ul), the marker *Ppa-egl-20*::*TurboRFP* (10ng/ul) and genomic carrier DNA (60ng/ul)

from the recipient strain. To study the as-*eud-1* lnc RNA, we generated a 7.5 kb construct consisting of approximately 6.5 kb promoter element and the 860 bp cDNA fragment of as-*eud-1*. This construct was injected (2ng/ul) with the *Ppa-egl-20*::*TurboRFP* (10ng/ul) marker and genomic carrier DNA (60ng/ul) of *P. pacificus* RS2333 and *Ppa-eud-1(tu445)*, respectively. For all constructs, at least two independent transgenic lines were generated and transgenic animals were scored over multiple generations. Transgenic lines containing the as-*eud-1* lnc RNA yielded more than 90% male progeny and all lines were kept at least until the tenth generation. No embryonic lethality was observed in association with these transgenes. Transgenic males were crossed with *Ppa-pdl-1* (ref. 21) and wild-type hermaphrodites and cross-progeny was readily observed.

Statistical Analyses

All phenotypic data show Eu frequency calculated from total individuals screened. Total sample size is illustrated on graphs. Significant differences were tested by Fisher's exact test. For the expression data we performed Kruskal-Wallis test. All Statistical analyses were implemented in the program Statistica v. 9 (Statsoft).

Western blotting and antibodies

Proteins were extracted from mixed stage cultures. Concentration was determined by Neuhoff's Dot-Blot assay[25]. Proteins were equally loaded and separated in polyacrylamid gels. Proteins were transferred to PVDF transfer membrane and incubated overnight with primary antibodies (Stable2), and were then incubated for an hour in secondary antibodies (Anti-rabbit IgG, HRP-linked Antibody, Cell Signaling Technology, Cat. #7074S and Anti-mouse IgG, HRP-linked Antibody, Cell Signaling Technology, Cat. #7076S). The detection was done by Bio-Rad Clarity western ECL substrate using Peqlab FUSION Xpress multi-imaging system.

Single molecule RNA FISH

Single molecule RNA FISH was performed using a protocol described earlier for *C. elegance*[27]. Biosearch Technologies Stellaris FISH online platform was

used to design and order *eud-s* and *as-eud-1* probes. They were coupled with Quasar 670 and TAMRA fluorescent dyes respectively. Image acquisition was performed on Leica SP8 confocal system using settings to maximize detection of fluorescent dyes. Image J software was used for Image analysis.

Sequence Vouchers

Sequences for *Ppa-lsy-12* and *Ppa-as-eud-1* have been deposited to GenBank. Accession numbers will be available soon.

REFERENCES

22. Srinivasan, J. *et al.* A bacterial artificial chromosome-based genetic linkage map of the nematode *Pristionchus pacificus*. Genetics *162*, 129-134 (2002).

23. Srinivasan, J. *et al.* An integrated physical and genetic map of the nematode *Pristionchus pacificus*. Mol. Genet. Genomics *269*, 715-722 (2003).

24. Trapnell, C., Roberts, A., Goff, L., Pertea, G., Kim, D., Kelley, D.R., Pimentel, H., Salzberg, S.L., Rinn, J.L. & Pachter, L. (2012). Differential gene and transcript expression analysis of RNA-Seq experiments with TopHat and Cufflinks. Nature Protoc. *7*, 562-578.

25. Neuhoff, V., Philipp, K., Zimmer, H.-G. & Mesecke, S. A simple, versatile, sensitive and volume-independent method for quantitative protein determination which is independent of other external influences. *Hoppe-Seyler's Z. Physiol. Chem.* **360**, 1657-1670 (1979).

26. Schuster, L. N. & Sommer, R. J. Expressional and functional variation of horizontally acquired cellulases in the nematode *Pristionchus pacificus*. *Gene*, 506, 274-282 (2012).

27. Ji, N. & van Oudenaarden, A. Single molecule fluorescent *in situ* hybridization (smFISH) of *C. elegans* worms and embryos, Wormbook, ed. The *C. elegans* Research Community, doi/10.1895/wormbook.1.153.1, http://www.wormbook.org. (2012).

Table S1 List of gene specific primers of *lsy-12* 3' and 5' RACE

CDNA END	PRIMER	OLIGO
3'	Co6GSP2	GCCGTGTTTGTCGCCGCAGTACGCGAGA
	Co6NGSP2	GAGGGCGCGAGCAGCAGCAATGAGGACG
	Co6.1GSP2	GAAATCGCGGCGGACACGGGCATCTCTC
	Co6.1NSP2	GGCGCTCGGGATGCTCGTCAAGAGCGAG
	Co6.2GSP2	GCTAAATTTTGGCATGGCCCCTCACTCG
	Co6.2NGSP2	GCGGCGGTATGCAGTCCACGCCGGGCAG
5'	Co6GSP1	GTACTTGAGGCAGAACTCGCAGATGTAC
	Co6NGSP1	GATAGGGCGCGGTGAACCACGTCTCCAT
	Co6.1GSP1	CCTCATATGCCGCCCACTGGTCCTGCGT
	Co6.1NGSP1	GCGTCGGCTCGTCACTGGGCGGCATCGA

Table S2 List of antibodies used in this study

Antibody	Host	Type	Company	Cat. #
Alpha tubulin	Mouse	mAb	SIGMA ALDORICH	F22168
H3K4me3	Mouse	mAb	Diagenenode	C15200152
H3K4me2	Rabbit	pAb	Diagenenode	C15410035
H3K4me1	Mouse	mAb	Diagenenode	Mab-150-050
H3K9ac	Mouse	mAb	Diagenenode	Mab-185-050
H3K27ac	Mouse	mAb	Diagenenode	C15200184
H2Bub	Mouse	mAb	Active Motif	39623, 39624
H2Aub	Mouse	mAb	Millipore	05-678
H4K20me1	Rabbit	pAb	Abcam	9051

Table S3 List of primers used for as-*eud-1* transcript identification

Primer	Oligo
Co10F3	GGAAGACTTCCGCAAGAAGCATGC
Co10R6	CTCGAGTGTTCATTCCTCAGGACATTG
Co10asFn	GAAGAACAAAACACACGGAGAAG
Co10asRn	ACTACCGAAAGACGAGACTACAATG
Co10F4	AGGGCCGAGTGGGCTACAAGGG
Co10R5	CCTACTACATCTCCACGCAGTGATC

Supplementary Figure Legends

SFig. 1: Mapping, gene structure and rescue of *P. pacificus lsy-12*. a, Genetic map showing the tip region of chromosome IV. **b,** Exon – intron structure of *Ppa-lsy-12* deduced from cDNA cloning and sequencing and molecular nature of mutations. The various shown isoforms are ordered by length. Note, that this list represents just a subset of the observed isoforms. Like in *C. elegans*, multiple isoforms exist and more than 50 exons are predicted for the *Ppa-lsy-12* gene. The successful rescue experiment indicated below were carried with a genomic fragment of approximately 17 kb covering 20 predicted exons as indicated by the arrow above the isoforms. **c,** Rescue experiment of the vulva defect of *tu319*. In wild type hermaphrodites, three vulval precursor cells are induced to form vulval tissue as indicated by an induction index of 3. *tu319* mutant animals have an average induction of 0.97 cells that is rescued to an average induction of 2.7 cells by an array carrying multiple copies of *Ppa-lsy-12*. Only one transgenic line was tested for the rescue of the vulval defect. **d,** Rescue experiment of the mouth-form defect of *tu319*. *tu319* animals are 2% Eu, whereas wild type hermaphrodites are 90% Eu under the same cultural conditions. Three independent transgenic lines (#1-#3) carrying multiple copies of *Ppa-lsy-12*, show 60-70% Eu animals as indicated by the analysis of more than 100 animals. Data are represented as the total Eu frequency.

SFig. 2: Histone modification of additional histone markers and *eud-1* expression in adult animals. a, *Ppa-lsy-12* and *Ppa-mbd-2* mutants do not result in obvious histone modification defects in several other tested histone marks. Here we show four representatives for ubiquitination (ub) and mono-methylation (me1). **b,** qRT-PCR experiments reveal down regulation of *eud-1* expression in *Ppa-lsy-12* and *Ppa-mbd-2* mutants relative to wild type in adult worms. *$p<0.05$ and ***$p<10^{-5}$, Kruskal-Wallis test.

SFig. 3: Nucleotide sequence of the as-*eud-1* transcript as obtained in RT-PCR experiments.

SFig. 4: Fluorescent in situ hybridization (FISH) of *eud-1*. FISH probes were designed as described in the Methods section. Photographs shown in a-c show the same adult animal using DAPI **a**, *eud-1* sense probes **b,** and a merger of both and differential interference contrast (DIC) microscopy **c,** *eud-1* sense probes stain several head neurons, two of which are clearly visible in this plane of focus. This pattern was highly consistent among hermaphrodites and males. In similar experiments using as-*eud-1* probes, no single was detected above background levels.

SFig. 5: Sequence conservation of the *eud-1* and as-*eud-1* locus between *P. pacificus* and its sister species *P. exspecatus*. The panel shows the percentage of sequence identity, which is well above 80% at the complete locus including antisense reads and introns.

a

S210 L106 L44

Chr. IV

L100 S73 L61

b

1K 2K 5K 6K 7K 8K 9K 10K 11K 12K 13K 14K 15K 16K 17K 18K 19K 20K 21K 22K 23K 24K 25K

tu319 G to A

tu30 G to A

tu18 598bp deletion

intron

utr

exon

deletion

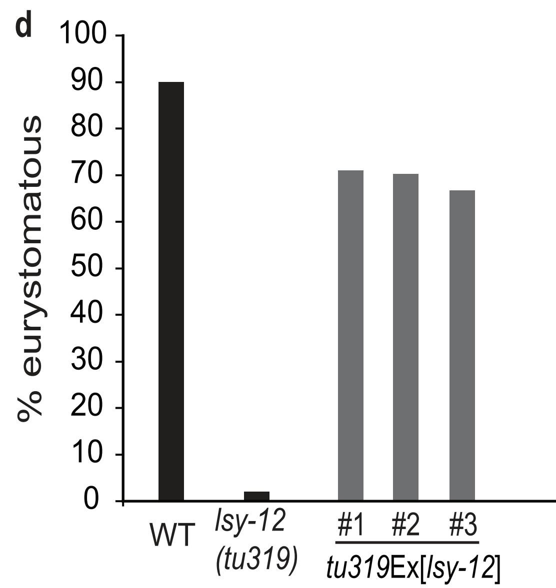
MYST Domain

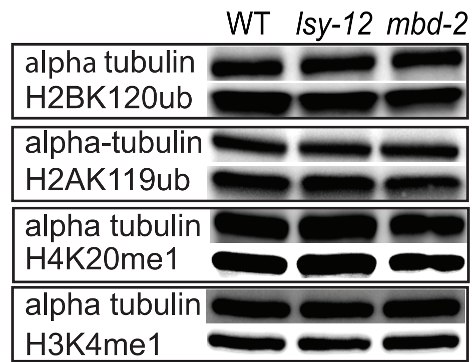
2K

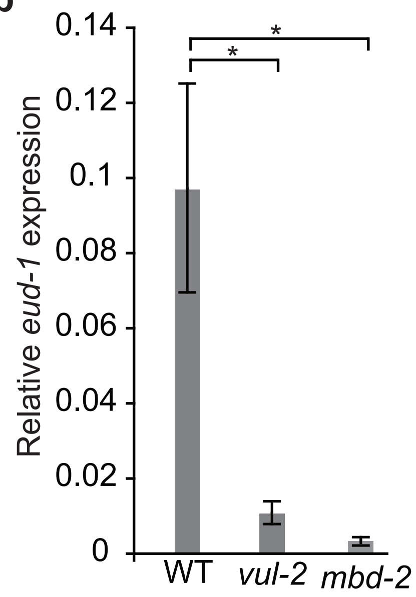
c

	WT	*lsy-12 (tu319)*	*tu319* Ex[*lsy-12*]
induction index	3	0.97	2.7
number of animals	many	72	10

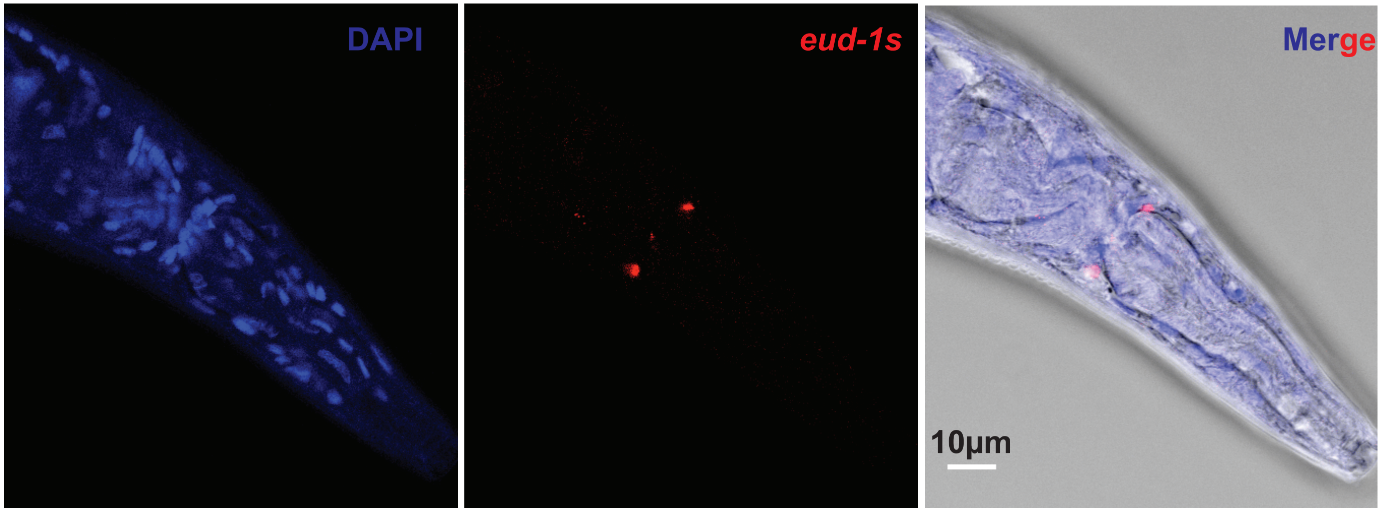
d

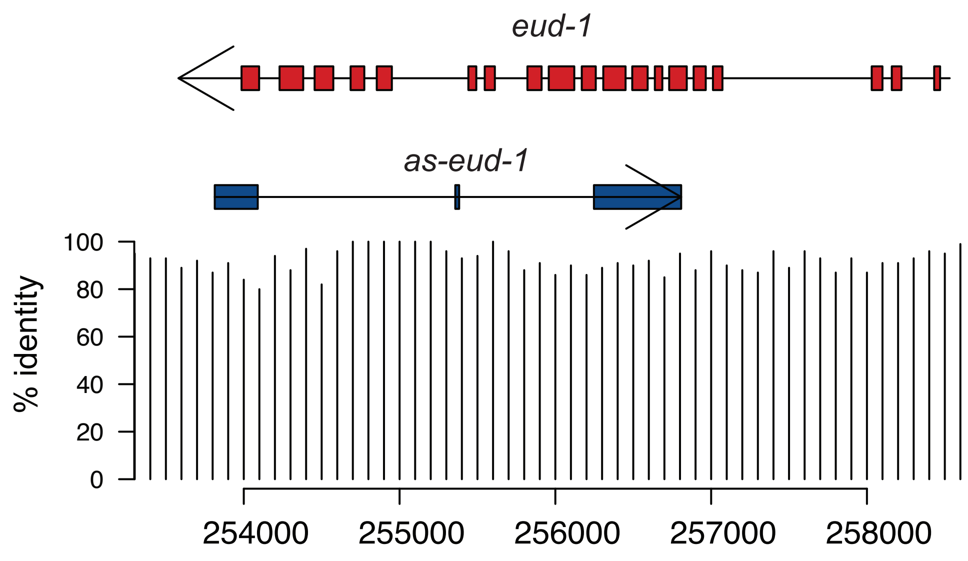



a
WT
lsy-12
mbd-2
alpha tubulin
H2BK120ub
alpha-tubulin
H2AK119ub
alpha tubulin
H4K20me1
alpha tubulin
H3K4me1


b
Relative eud-1 expression
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
*
*
WT
vul-2
mbd-2

5’ggaagacttccgcaagaagcatgcaaacactacattctaaatgatacaagagatttaacaatttactataataaatgtccagttg
aagaacaaaacacacggagaaggttgtgaagggtttgcgaaagaactgatggaagcgagataaatcgttaggtatatttattctta
ttcaagctgatcgtattcttgatcactccagttgagttcttccttctcgagcatggctcttcgaagcgcgcgctggaatcgatcactgc
gtggagatgtagtagggtcgtaaaacaacgctaggtgggaccagggccgagtgggctacaagggaatcattatttgggatttta
atactagcaaattagtacctcggcttttctctggtgaatgaagaacttaaaatcatcaaccaaattctgagtcatatcgtcgaatatca
tgggttgttgcaccaactcatcaccgtcatacaagaagcacttggttccattgggaccatcagggaacattgtctaaacaatctcga
aaattggtgttggtagatttttcagtaccttagtggtatcgcactcccatacatttgtgaagggaaggttgactcccacgtattgaaaa
ccacgacgggaaggcaaatgtactccgtcagtactggttctgaagcataaccatttaaagtgctgataattacaatttactaactctt
cattgattccgagatgccatttgccgatcattcctgttgcgtaacctgaatttgcaattatttagatgtattcaatttcaattcccacctcg
ttcttcgaacatctcggccattgtagtctcatctttcggtagtcctccaatgtcctgaggaacgaacactcgag3’


DAPI
eud-1s
Merge
10μm


eud-1
as-eud-1
% identity
100
80
60
40
20
0
254000
255000
256000
257000
258000