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

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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

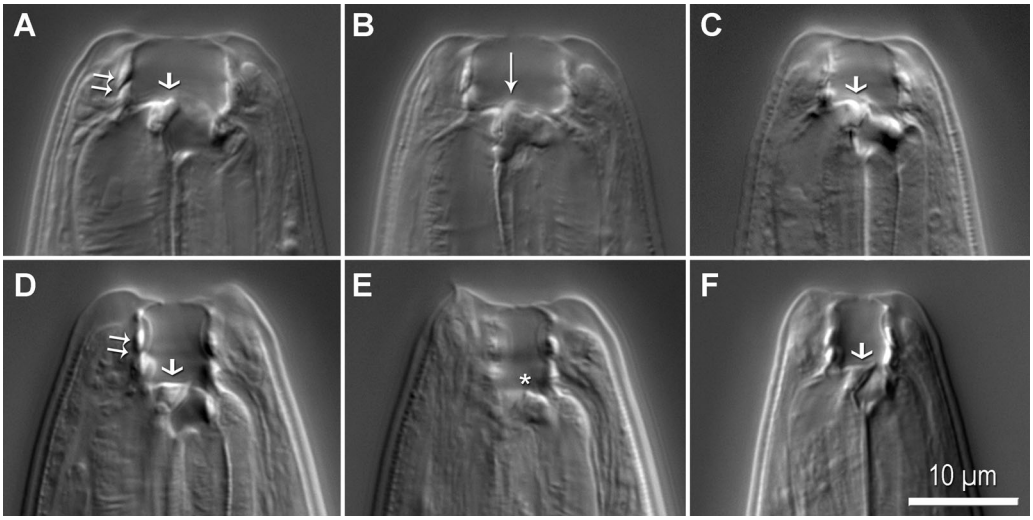

Fig. 1. DIC micrographs of the dimorphic stoma (mouth) of *Pristionchus pacificus*. All images are at same scale. Dorsal is left in all images. (A and B) Eurystomatous hermaphrodite in the sagittal and right sublateral planes, respectively. (C) Eurystomatous male in the sagittal plane. (D and E) Stenostomatous 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*)
Maternal phenotype	Total	86.33 ± 2.55	19 (1352)	83.24 ± 3.06	28 (1482)	21.28 ± 3.87	28 (580)
	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

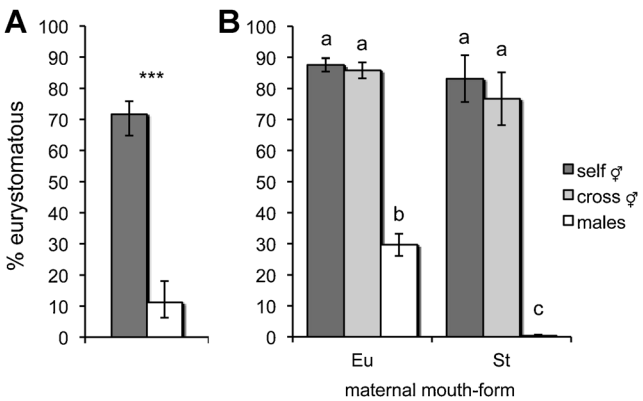

Fig. 2. The mouth-form phenotype of *P. pacificus* by sex, parentage, and maternal phenotype. (A) The total eurystomatous frequencies of spontaneous males (open bars) that is males produced by X-chromosome non-disjunction, and of hermaphrodites (dark gray) occurring in the same culture populations as 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.

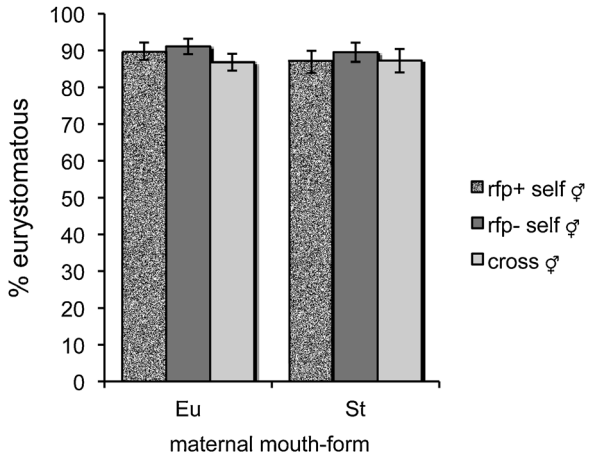

Fig. 3. The effect of paternity on hermaphroditic progeny as tested by crosses with a *Ppa-egl-20*::rfp reporter. The mean eurystomatous frequencies of *Ppa-egl-20*::rfp self-hermaphrodites (textured), wild-type self-hermaphrodites (dark gray), and *Ppa-egl-20*::rfp cross-hermaphrodites (light gray) 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,

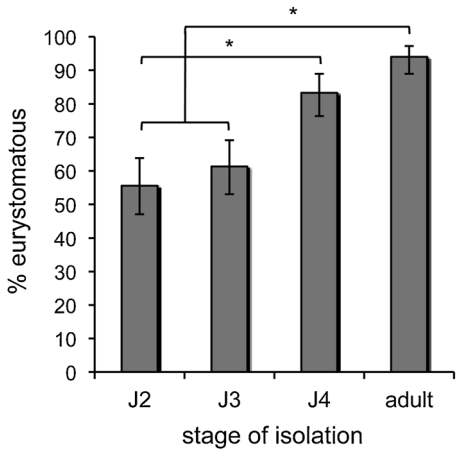

Fig. 4. The mouth-form phenotype of *P. pacificus* hermaphrodites when isolated from populations at different life-stages. Individuals were transferred at one of the three post-eclosion juvenile stages (J2–J4) or allowed to reach the adult stage together with conspecifics. Total eurystomatous frequencies 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.

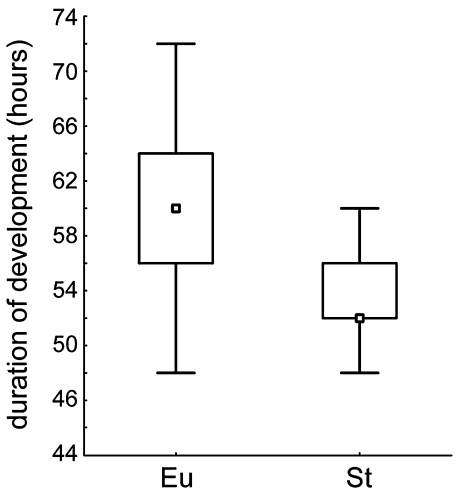

Fig. 5. The duration of post-embryonic development in the two mouth forms of *P. pacificus* at 20°C. Individuals were isolated as hatchlings ($\leq$2 h old) and their development tracked every 4 h until reaching the adult stage. Upon becoming adults, their mouth-form phenotype was recorded. Box plots 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.

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