LETTERS

Co-option of the hormone-signalling module dafachronic acid–DAF-12 in nematode evolution

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Morphological novelties are lineage-specific traits that serve new functions[1,2]. Developmental polyphenisms have been proposed to be facilitators of phenotypic evolution, but little is known about the interplay between the associated genetic and environmental factors[3–11]. Here, we study two alternative morphologies in the mouth of the nematode *Pristionchus pacificus* and the formation of teeth-like structures that are associated with bacteriovorous feeding and predatory behaviour on fungi and other worms[12–16]. These teeth-like denticles represent an evolutionary novelty, which is restricted to some members of the nematode family Diplogastridae but is absent from *Caenorhabditis elegans* and related nematodes[14]. We show that the mouth dimorphism is a polyphenism that is controlled by starvation and the co-option of an endocrine switch mechanism. Mutations in the nuclear hormone receptor DAF-12 and application of its ligand, the sterol hormone dafachronic acid, strongly influence this switch mechanism. The dafachronic acid–DAF-12 module has been shown to control the formation of arrested dauer larvae in both *C. elegans* and *P. pacificus*, as well as related life-history decisions in distantly related nematodes[17–20]. The comparison of dauer formation and mouth morphology switch reveals that different thresholds of dafachronic acid signalling provide specificity. This study shows how hormonal signalling acts by coupling environmental change and genetic regulation and identifies dafachronic acid as a key hormone in nematode evolution.

Pristionchus nematodes have a necromenic association with scarab beetles (Fig. 1a, b). They rest as dauer larvae on the insect and resume development in the beetle carcass to feed on the growing microbes[15]. Besides bacteria, *P. pacificus* can feed on fungi and other nematodes using its teeth-like denticles[12,14] (Fig. 1c). For example, *P. pacificus* can actively kill *C. elegans* and use it as food source (see Supplementary Video 1). The mouth of *P. pacificus* occurs as a dimorphism, the eurystomatous (EU) and stenostomatous (ST) form (Fig. 1d–f). The dorsal left denticle of EU worms is bigger and has a claw-like shape (Fig. 1e) and EU worms have an extra right ventral denticle (Fig. 1d) that is not present in ST worms[12,14] (Fig. 1f). In addition, there are quantitative differences, where EU worms have a shallow and broad cavity, whereas ST worms have a narrow and deeper cavity[14]. The mouth morphology of an individual nematode is irreversible and is executed at the final moult from the J4 larval stage to adulthood[12,14].

First, we tested if the mouth dimorphism in *P. pacificus* is a polyphenism, that is, that changing environmental conditions determine alternative developmental fates[7], or the result of genetic polymorphism. Using an inbred line regime we selected for ST or EU individuals for 10 generations. No differences were found between different selection pressures, showing that the mouth dimorphism is indeed a polyphenism ($P = 0.094$) (Fig. 2a).

Next, we determined which environmental factors have an influence on the mouth switch mechanism. Starvation in early larval development resulted in a significant increase of EU worms ($P < 0.001$) (Fig. 2b). Specifically, 84% of worms starved as J2 larva developed EU morphology after recovery, when compared to 32% in non-stressed animals (Fig. 2b). In *C. elegans* and *P. pacificus*, starvation and overcrowding are environmental conditions that result in the formation of dauer larvae, an alternative, stress-resistant larval stage with a highly extended life span that also represents an environmentally induced polyphenism[19,21–23]. Population density is one of the most important factors influencing dauer arrest. In *C. elegans* population density is signalled by a pheromone consisting of a blend of chemicals[24–27]. Similarly, extracts of *P. pacificus* liquid cultures contain a pheromone that induces dauer formation in *P. pacificus*, but not in *C. elegans*[19].

To study if population density affects the mouth polyphenism we tested the influence of *P. pacificus* pheromone on the buccal morphology switch. *P. pacificus* pheromone had a strong impact on mouth morphology in that 70% of the animals had the EU fate ($P < 0.001$) (Fig. 2b). In contrast, *C. elegans* pheromone had no influence on the *P. pacificus* mouth form decision, indicating species-specificity in signal recognition ($P = 0.67$) (Fig. 2b). These experiments indicate that *P. pacificus* mouth and dauer formation represent morphological and life cycle responses to similar environmental stimuli.

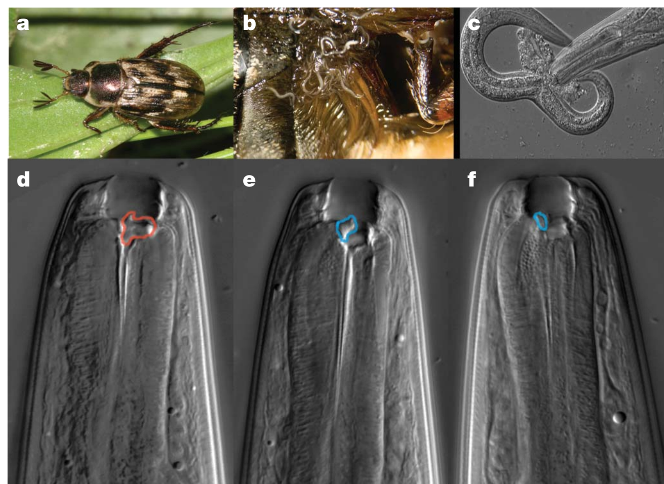

Figure 1 | Ecology and mouth dimorphism in *Pristionchus pacificus*. **a**, *Exomala orientalis* is one scarab beetle host of *P. pacificus* (photo: M. Herrmann). **b**, Worms resume development on beetle carcass (photo: D. Bumbarger). **c**, *P. pacificus* feeds on *Caenorhabditis elegans* larva (photo: A. Weller). **d**–**f**, *P. pacificus* can have one of two distinct morphologies, eurystomatous (**d**, **e**) or stenostomatous (**f**). Eurystomatous worms have a subventral right denticle (**d**, red outline), and the dorsal left denticle (**e**, **f**, blue outline) is bigger and claw-like unlike in stenostomatous worms.

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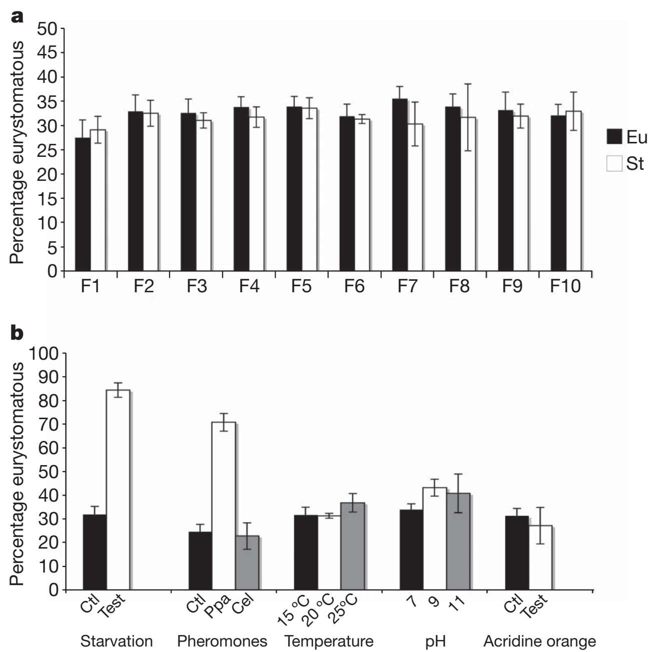

Figure 2 | Mouth dimorphism is an environmentally induced polyphenism. **a**, Selection of inbred lines for eurystomatous (EU) or stenostomatous (ST) morphologies show no differences between selection pressures ($P = 0.009$). **b**, Worms were challenged with different environmental factors. Starvation ($P < 0.001$) and *P. pacificus* pheromone ($P < 0.001$) produced an increase in EU worms. Data represents means ± s.d. (95%). Ctl, control; Cel, *C. elegans*, Ppa, *P. pacificus* pheromone.

Next, we wanted to know if dauer formation and the mouth dimorphism share common genetic and molecular control mechanisms. In *C. elegans*, pheromonal cues are processed through several signalling pathways, which regulate the activity of a class of steroidal hormones, Δ4- and Δ7-dafachronic acid (DA)[17,18]. The decrease of DA shifts the nuclear hormone receptor DAF-12 from its ligand-bound form to a ligand-free form, thereby specifying dauer fate[17,18]. Recent studies indicate that the DA–DAF-12 module is evolutionarily conserved and that DA acts as a pharmacological activator that can regulate dauer formation in both *C. elegans* and *P. pacificus*[19] (Fig. 3a).

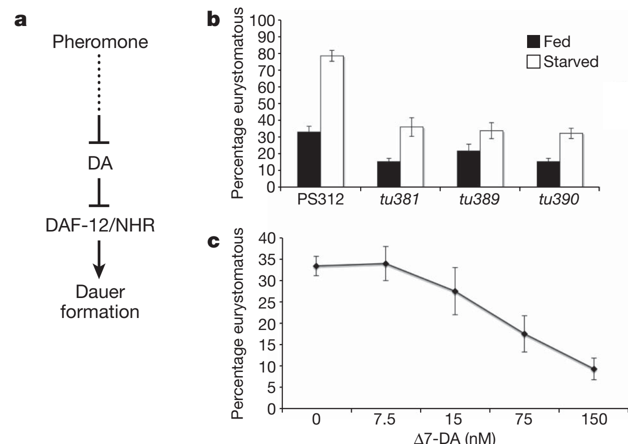

Figure 3 | The dafachronic acid–DAF-12 endocrine signalling module was co-opted for the mouth polyphenism. **a**, Scheme of *P. pacificus* dauer pathway. **b**, All three available alleles of *Ppa-daf-12* show a significant reduction in number of eurystomatous worms ($P < 0.001$), but they still respond to starvation ($P = 0.009$; 0.016; 0.009). **c**, Application of Δ7-DA reduces number of EU in a dose-dependent manner ($P < 0.001$). Data represents means ± s.d. (95%). NHR, nuclear hormone receptor.

To study if the dauer regulatory cascade is involved in the buccal cavity decision in *P. pacificus*, we analysed dauer formation-defective (*daf-d*) mutants and applied Δ7-DA in pharmacological assays. Mutants in the nuclear hormone receptor *Ppa-daf-12* showed a strong decrease in EU worms in all three available alleles ($P = 0.009$) (Fig. 3b). However, even these strong loss-of-function mutations in *Ppa-daf-12* that abolish dauer formation did not completely suppress the formation of the EU morph (Fig. 3b). Application of Δ7-DA had a concentration-dependent influence on the mouth decision. *P. pacificus* responded to an increased concentration of Δ7-DA with a decrease in the number of EU worms in a dose-dependent manner ($P < 0.001$) (Fig. 3c). This is consistent with the phenotype of the *Ppa-daf-12* mutants (Fig. 3a, b). Furthermore, application of Δ7-DA at different time points in larval development indicated that the DA–DAF12 module can influence the mouth decision only through the J2 larval stage, but not in later stages ($P < 0.001$) (Supplementary Fig. 1a). Together, these experiments indicate that DA–DAF12 regulates the buccal cavity switch mechanism and that this endocrine signalling system was co-opted for a new function during nematode evolution. Interestingly, the direction of response to both environmental cues and hormonal signalling has been conserved between the regulation of dauer formation and the mouth form dimorphism.

Given the co-option of the DA–DAF-12 module, how does *P. pacificus* regulate the specificity of its phenotypic responses? To address this question we first applied pheromone extracts at different concentrations. The *P. pacificus* response to pheromone extracts is dose-dependent with higher numbers of EU worms ($P < 0.001$) and dauers formed ($P = 0.006$) in response to increased pheromone concentrations (Fig. 4a). However, the concentration of pheromone required for increasing EU worms is considerably lower than the one required for efficient dauer formation. Specifically, 1.5% (v/v) of pheromone extract in agar plates was sufficient to obtain more than 80% of EU adult worms (Fig. 4a). In contrast, even the application of the highest concentration of pheromone extract, 12% (v/v), resulted in the formation of only 15% dauer larvae (Fig. 4a). We can rule out the possibility that differences in timing of sensitivity are responsible for the distinct developmental decisions because both decisions have their critical periods during the J2 stage (Supplementary Fig. 1a, b). Thus, different signalling thresholds during dauer formation and mouth decision control the independent developmental response to a conserved physiological regulator. Interestingly, the increase of EU worms was obtained only when animals had gone through the direct life cycle, whereas worms that went through the dauer stage became almost exclusively ST on recovery ($P < 0.001$) (Fig. 4b). That can be the result of either developmental or environmental constraints, because under laboratory conditions dauer larvae resume development only in the presence of bacterial food[19].

Second, we searched for interactions between genetic and environmental factors to test whether high pheromone concentrations and/or starvation influence the mouth decision in *Ppa-daf-12* mutants. We found that in *Ppa-daf-12* mutants pheromone application had no influence on mouth form ($P = 0.86$) (Fig. 4c). This indicates that the pheromone acts completely through the DAF-12 pathway. In contrast, if *Ppa-daf-12* mutants are starved at the J2 stage, a significant increase in the number of EU animals was obtained ($P < 0.001$) (Fig. 3b). We conclude that pheromone signalling acts completely through DAF-12, whereas starvation acts through both DAF-12-dependent and DAF-12-independent pathways.

In a final experiment, we tested whether the application of Δ7-DA could suppress the influence of pheromone on both the mouth switch and dauer formation. We found that higher concentrations of Δ7-DA are necessary to suppress the effect of pheromone in mouth polyphenism than in dauer formation (Fig. 4d). Specifically, a concentration of 250 pM of Δ7-DA was sufficient to completely suppress dauer formation, whereas a 25 nM concentration of Δ7-DA was necessary to bring the number of EU worms back to levels comparable to a pheromone-free environment ($P < 0.001$) (Fig. 4d).

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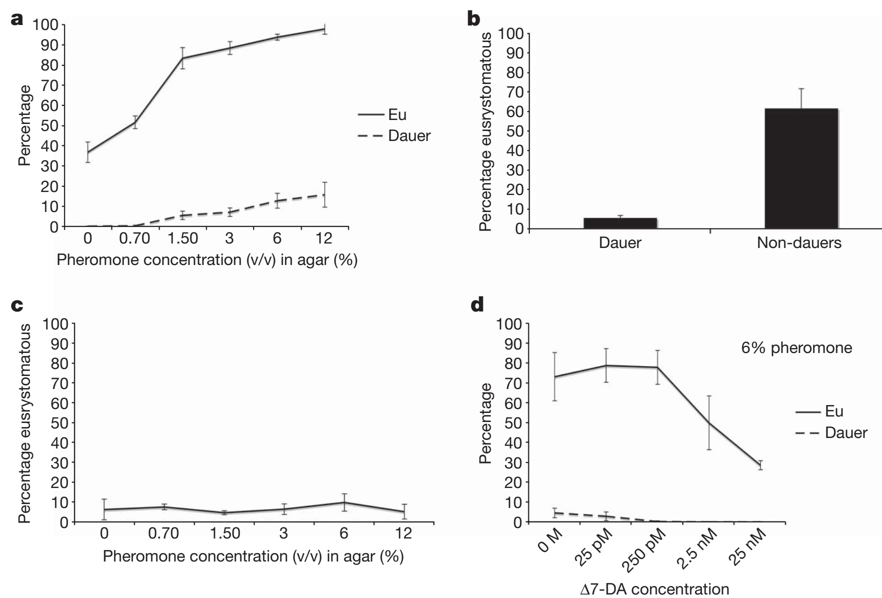

Figure 4 | Different pheromone and hormone thresholds for dauer and mouth formation provide specificity in the phenotypic responses. a, Mouth switch requires lower concentrations of *P. pacificus* pheromone than dauer formation for a significant response. **b**, Recovered dauers have a different mouth fate than animals that do not undergo dauer stage ($P < 0.001$). **c**, *Ppa-daf-12* worms mouth switch mechanism does not respond to *P. pacificus* pheromone ($P = 0.8689$). **d**, The mouth switch mechanism requires a much higher concentration of Δ7-DA than dauer formation to suppress pheromone effect. Data represents means ± s.d. (95%).

Taken together, these three sets of experiments indicate that (1) pheromone influences the mouth polyphenism through a developmental pathway that includes the DA–DAF-12 module; (2) the dauer and buccal cavity switch respond to distinct thresholds of both pheromone and hormonal signalling and (3) there is an alternative developmental pathway that influences the buccal cavity decision in response to starvation.

The integration of genetic and environmental factors in the regulation of polyphenisms remains key for the understanding of phenotypic evolution[4–11]. The study of the mouth polyphenism in *P. pacificus* reveals a central role for hormonal signalling. First, the mouth switch mechanism is regulated by an endocrine hormone signalling module and second, this hormone module has been co-opted to serve different functions in nematode evolution. Hormones are common regulators of post-embryonic development in animals and plants[28]. At the same time hormones are sensitive to environmental change and can couple information of the environment to genetic regulation[28]. In our study, information of population density, which is mediated by pheromone concentration, is coupled to the transcription factor DAF-12 through the sterol hormone DA. The comparison between mouth polyphenism and dauer formation showed that distinct thresholds provide specificity in developmental decisions. The mouth polyphenism requires less pheromone and is less sensitive to the Δ7-DA hormone compared to dauer formation. Therefore, both endocrine hormone signalling and the pheromone function in a concentration-dependent manner. With a common switch mechanism, the dosage of pheromone and hormonal signals is crucial for independent developmental decisions.

We propose a central role for the DA–DAF-12 signalling module in the evolution and diversification of nematodes. Recent studies showed that DA is involved in the regulation of the life cycle of parasitic nematodes[19,20]. Application of DA to early larvae of *Strongyloides* and *Ancylostoma* parasites prevented the formation of infective juveniles, indicating that DA is broadly used as a developmental regulator[19,20]. A similar case is seen in insects, where juvenile hormone (JH) and ecdysteroids are directly involved in growth, reproduction, behaviour, moulting and diapause and where these same hormones are also responsible for the regulation of polyphenisms[4,10,28,29]. Similarly, in plants jasmonate signalling has been co-opted for various environment-sensitive plastic responses[30]. Therefore, the co-option of highly conserved endocrine signalling mechanisms is a repeated feature of phenotypic evolution.

METHODS SUMMARY

All wild-type worms were *P. pacificus* reference strain PS312. *Ppa-daf-12* mutants were previously obtained by ethyl methanesulphonate mutagenesis, cloned and described previously[19]. In all experiments phenotypes were scored using a Zeiss Discovery V12 stereomicroscope. Three markers allow identification of EU and ST worms: (1) head shape, EU worms have a straight head in comparison to ST worms that have a funnel-like head shape; (2) buccal cavity shape, EU worms have a broad and shallow buccal cavity, whereas ST worms have a comparatively narrow and deeper cavity; (3) colouration at the base of buccal cavity, EU worms have a dark stripe at the base of their buccal cavity resulting from extra denticle structures. This stripe is weaker or totally absent in ST worms. All worms that could not be scored as either EU or ST were ruled out. Occasionally, some intermediate forms can be observed through observation by Nomarski microscopy, but not by using the Discovery V12 stereomicroscope. In these cases, the claw-like tooth is not clearly visible. Although the number of males is normally very low, <1%, males were excluded from the analysis to avoid sex-related effects.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions G.B. performed all experiments except the experiments described in Supplementary Fig. 1b, which were performed by A.O.; G.B., A.O. and R.J.S. designed the experiments and wrote the manuscript.

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METHODS

Culture conditions. With the exception of pheromone experiments all worms were kept in 6 cm nematode growth media (NGM) agar plates and fed with 250 µl of *Escherichia coli* strain OP50. Except for temperature experiments worms were kept at 20 °C and approximately 50% humidity. All plates that showed any signs of contamination (fungal, bacterial or other) were excluded from the analysis.

Selection experiments. Selection for EU and ST: 20 selection-inbred lines (SIL) were generated from a single population. For 10 of those, one EU hermaphrodite was selected in each generation for 10 generations. The adult progeny of each selected worm was scored after 5 days. For the remaining 10 SILs, ST hermaphrodites were selected.

Environmental challenges. Starvation: five J4 worms were isolated in one plate. After 10 days all food was gone and the F2 generation arrests its development at the J2 larval stage. These worms are then transferred to a new plate and fed to develop into adults and scored after 2–3 days. In control fed worms, five J4 worms were placed in a plate and their progeny scored.

Pheromone: both *P. pacificus* and *C. elegans* pheromones extracts were obtained using a protocol described previously[19]. The worms were cultured in 3.5 cm plates with 3 ml NGM medium without cholesterol or peptone and fed with 40 µl of 2% dead *E. coli* OP50 (ref. 19). To each plate 100 µl of water, *P. pacificus* pheromone or *C. elegans* pheromone were added. For each plate 5–10 adult hermaphrodite worms were allowed to lay eggs for 3 h and then killed. Scoring was performed after 4 days.

Temperature: adult hermaphrodites were singled and kept at 15 °C, 20 °C or 25 °C. Scoring of progeny was performed 7, 5 and 3 days after singling, respectively.

pH: NGM medium was melted and mixed with HCl or NaOH until it reached a pH of 7, 9 or 11. Hermaphroditic adults were singled into this plates and progeny was scored 5 days later.

Acridine orange: acridine orange was reported to have an effect on dimorphic worms[12]. We applied 100 µl of a solution of acridine orange in water (1:10,000) (w/v).

Ppa-daf-12. *Ppa-daf-12* mutants of all available strains were singled and progeny scored after 5 days.

Δ7-DA assays. A stock solution of 15 µM Δ7-DA in ethanol was prepared. Treatments differed by applying 100 µl of this stock solution at concentrations of 1:1, 1:2, 1:10 and 1:20 (v/v) in ethanol. The control consisted of the application of 100 µl of 100% ethanol. Pharmacological applications were performed at day 0 (when hermaphrodites were singled) and day 3. Scoring was performed at day 5.

For the stage-dependent application of Δ7-DA we used the starvation protocol, applying: (1) 100 µl of ethanol in first plate at days 0, 3, 6 and 9 and also in plate 2; (2) 100 µl Δ7-DA 7.5 µM at the same time points; (3) 100 µl Δ7-DA 7.5 µM in plate 1 and 100 µl of ethanol in plate 2; (4) 100 µl of ethanol in plate 1 and 100 µl Δ7-DA 7 5 µM in plate 2.

Pheromone interaction assays. Concentration: same protocol as previously was applied only with different concentrations of pheromone extract: 0:200 µl water; 25:175; 50:150; 100:100; and 200:0. Dauers were scored after 3 days as the number of dauer over the total number of worms. EU and ST forms were scored at day 4 as number of EU over total number of adults. This protocol was applied to both wild-type and *Ppa-daf-12* worms.

Dauer assay: large numbers of dauers were obtained through the following protocol. Worms from previously prepared cultures were washed into 10-cm wet plates. Humidity was kept high throughout the 10 days by keeping wet paper towels in the boxes where plates were kept. After 10 days dauers and mixed staged worms were separated through this protocol. Plates were washed with a 0.2% (v/v) solution of Triton/M9, the medium was then centrifuged at 1,000*g* for 15 min. The pellet was resuspended with 20 ml Ficoll (20% (v/v) in M9/Triton) and incubated for 5 min at room temperature. The solution was separated into two 50 ml Falcon tubes and filled with Triton/M9 and centrifuged at 1,500*g* for 10 min. The pellets were recombined into one Falcon tube, resuspended in Triton/M9 and centrifuged at 2,000*g* for 20 min. The pelletd was resuspended with 30 ml sucrose (50% (v/v) in Triton/M9) and 20 ml Triton/M9, and centrifuged at 1,000*g* for 10 min.

After this procedure, dauers could be found in supernatant whereas mixed stages were in the pellet. The liquid and solid phases were separated and both were extensively washed with M9.

Dauers and mixed stage worms were then plated. Adults were scored after 2–4 days.

Timing of dauer induction: for dauer formation assays with the conditioned medium, the supernatants of *P. pacificus* PS312 liquid culture were prepared and filtered as described previously[1]. Bleached eggs of *P. pacificus* were allowed to hatch in the conditioned medium or control medium and were subsequently transferred to medium containing 0.1 (w/v) *E. coli* OP50. Fisher's exact test was used to test independence. Error bars denote 95% exact binomial confidence intervals. Statistical analysis was performed with the program R.

Pheromone-Δ7-DA interaction assay: conditions were identical to previous pheromone assays. Control plates had 200 µl of water. All other plates had 100 µl of *P. pacificus* pheromone extract. A 7.5 µM Δ7-DA solution in water was prepared. Different treatments were prepared with 100 µl of this solution in water: 1:1,000, 1:100, 1:10, 1:1 and only water for the 0 Δ7-DA. Dauers were scored after 3 days, whereas adults were scored after 4 days.

Statistical analysis. For each experiment one plate is one replicate. The percentage of EU worms in each plate is the result. For statistical analysis all data was transformed by the ASIN method[31]. For all data sets ANOVA assumptions were graphically tested[31]. A two-way ANOVA was performed for the selection experiment. For *Ppa-daf-12* mutants, Δ7-DA concentration and pheromone concentration experiments one-way ANOVA was performed. Bonferroni multiple comparison tests were performed for this data sets. All other data sets were tested using the non-parametric Kruskal–Wallis rank test[31]. Confidence intervals of 95% based on the standard deviation (s.d.) of the data were calculated for each data set. All statistical analysis was performed with Small Stata 9.2 software.

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