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A Conserved Endocrine Mechanism Controls the Formation of Dauer and Infective Larvae in Nematodes

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Summary

Under harsh environmental conditions *Caenorhabditis elegans* larvae undergo arrest and form dauer larvae that can attach to other animals to facilitate dispersal[1]. It has been argued that this phenomenon, called phoresy, represents an intermediate step towards parasitism[2,3]. Indeed, parasitic nematodes invade their hosts as infective larvae, a stage that shows striking morphological similarities to dauer larvae[1]. While the molecular regulation of dauer entry in *C. elegans* involves insulin and TGF-ß signaling[4-8], studies of TGF-ß orthologues in parasitic nematodes did not provide evidence for a common origin of dauer and infective larvae[9-14]. To identify conserved candidate regulators between *Caenorhabditis* and parasitic nematodes we used an evolutionary approach involving *Pristionchus pacificus* as intermediate. We show by mutational and pharmacological analysis that *Pristionchus* and *Caenorhabditis* share the dafachronic acid-DAF-12 system as core endocrine module for dauer formation. One of the dafachronic acids, Δ7-DA, has a conserved role in the mammalian parasite *Strongyloides papillosus* where it controls entry into the infective stage. Application of Δ7-DA blocks formation of infective larvae and results in the generation of free-living animals. The conservation of this small molecule ligand represents a fundamental link between dauer and infective larvae and might provide a general strategy for nematode parasitism.

Results and Discussion

In *C. elegans*, pheromonal cues that indicate overcrowding, high temperature or starvation are processed through several signaling pathways including insulin/IGF, TGFβ-like, and guanylyl cyclase pathways (Fig. 1A) [4-8,15-20]. This results in the decrease of a class of steroidal hormones, Δ4-DAfachronic acid and Δ7-DAfachronic acid (DAs) and shifts the nuclear hormone receptor DAF-12 from its ligand-bound form to a ligand-free form, which specifies the dauer fate [21-25]. DAF-12 is strictly required for *C. elegans* dauer development [26] and its systemic expression suggests a role in the specification of dauer fate at the level of individual tissues [24]. Despite the wealth of knowledge of dauer formation in *C. elegans*, the extent to which this paradigm applies to other nematodes is not comprehensively investigated. Pharmacological studies in *Ancylostoma* species, parasitic nematodes related to *C. elegans* (Fig. 1C), identified potential parallels in the recovery of infective and dauer larvae [27-29].

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However, little is known about mechanisms involved in the specification of cell fates during the formation of the infective larvae. Furthermore, studies on the ortholog of *daf-7* in *Ancylostoma* and other parasitic nematodes indicated substantial functional divergence during infective larvae formation [8-14]. Also, dauer pheromones of several rhabditid nematodes were found to be ineffective for *C. elegans* [15]. Only in the close relative *C. briggsae* several genes were found to play a similar role as in *C. elegans* [30].

Given the complexity of the dauer regulatory network and the limited functional conservation of some dauer control genes, we used an evolutionary approach to identify candidate genes that might be shared with parasitic nematodes. Specifically, we involved a distant relative of *C. elegans*, *P. pacificus* as an intermediate (Fig. 1C). *P. pacificus* is a genetically tractable system that shares many features with *C. elegans*, such as a short generation time, easy laboratory culture, and a complete genome sequence [31]. Forward and reverse genetic analysis in *P. pacificus* and the deep position of the last common ancestor of *Pristionchus* and *Caenorhabditis* makes *Pristionchus* an ideal model for exploring the evolution of developmental mechanisms [32,33]. In the wild, *Pristionchus* species associate with beetles as dauer larvae, which resume development only after the death of the beetle when they begin feeding on microbes on the carcass [34,35]. Thus, the dauer stage is important for the adaptation of *P. pacificus* to its ecological niche.

To explore evolutionarily conserved mechanisms of dauer formation, we first asked if a pheromone(s) controls dauer formation in *P. pacificus*. We found that supernatant prepared from liquid *P. pacificus* cultures induced eggs and J2 larvae to enter the dauer stage (data not shown). Next, we tested if *P. pacificus* and *C. elegans* employ different pheromones by preparing concentrated pheromone extracts from supernatants (Fig. 2A) [36]. In plates containing *P. pacificus* pheromone extracts, *P. pacificus* animals were induced to form dauer, whereas only few *C. elegans* N2 animals went into dauer ($p<0.01$). Similarly, *C. elegans* pheromone induced dauer formation in *C. elegans*, but not in *P. pacificus* ($p<0.01$). Thus, *P. pacificus* and *C. elegans* use distinct dauer pheromones, a result similar to previous studies with nematodes more closely related to *C. elegans* [15].

In *C. elegans*, depletion of cholesterol from culture medium induces dauer formation [37]. This is because *C. elegans* relies on an environmental source for the precursor of the DA hormone, which through binding to DAF-12 inhibits dauer formation. Under laboratory conditions, cholesterol in the medium serves as precursor of DA. To test if cholesterol plays a similar role in *P. pacificus* dauer formation, we examined the effect of cholesterol restriction in the dauer entry assay. When grown on an agar plate containing pheromone without added cholesterol, a significantly higher number of dauer larvae were formed than on plates with added cholesterol ($p<0.01$), suggesting that as in *C. elegans* sterol hormones might regulate dauer formation in *P. pacificus* (Fig. 2B).

To test if a DA-DAF-12 system controls dauer formation, we set out to isolate dauer formation defective (Daf-d) mutants in forward genetic screens in *P. pacificus*. To identify *Ppa-daf-12* alleles that would be epistatic to cholesterol depletion, we screened for Daf-d mutants using liquid medium without cholesterol. Three Daf-d mutants mapped to the *Ppa-daf-12* region of the genome and molecular analysis indeed showed that they carry a molecular lesion in *Ppa-daf-12* (Fig. S4). *Ppa-daf-12* encodes a nuclear hormone receptor, which shows >92% and 54% amino acid sequence identity to *Cel*-DAF-12, in the DNA binding domain (DBD) and ligand binding domain (LBD), respectively (Fig. S4B, C). Three segments of the DBD are important for DNA recognition, the proximal (P), distal (D), and direct repeat (DR) boxes, respectively [38,39]. All three segments are completely conserved, suggesting conservation of the DNA recognition specificity. The three mutations in *Ppa-daf-12* are nonsense and splice acceptor mutations that truncate the LBD and parts of the hinge region (Fig. S4A). None of

the *Ppa-daf-12* mutants formed dauer larvae on an agar plate containing dauer pheromone indicating that *Ppa*-DAF-12 is essential for dauer entry (Fig. 2C).

Next we wanted to know if DAs, the steroid compounds that serve as small molecule ligands for the DAF-12 receptor, are also conserved between *P. pacificus* and *C. elegans.* In *C. elegans,* DAs can rescue dauer formation constitutive (Daf-c) phenotypes resulting from mutations in multiple signaling pathways acting upstream of DAF-12 (ref. 5). In forward genetic screens for *P. pacificus* Daf-c mutants, we isolated two incompletely penetrant and two fully penetrant mutations. These mutations map to four separate chromosomal regions (Fig. S1). We tested if Δ4- DA and Δ7-DA could rescue these *Ppa*-Daf-c mutants. Administration of Δ7-DA strongly suppressed dauer formation in all four *P. pacificus* Daf-c strains $p<0.01$; Fig. 3A). Δ4-DA rescued two incompletely penetrant mutations ($p<0.01$ for both strains), but did not significantly affect the two fully penetrant mutations ($p=0.11$, and $p= 0.56$ for *Ppa-dfc-3* and *Ppa-dfc-4*, respectively). These results suggest that DA/DAF-12 is a conserved endocrine module and represents a candidate for conservation in parasitic species.

While many nematodes are obligate parasites with limited access for experimental studies, some species retain a free-living life cycle in addition to the parasitic life cycle. One such example is *Strongyloides papillosus*, a parasite of ruminants that can be cultured in rabbits under laboratory conditions [40]. Phylogenetically, *Strongyloides* is an outgroup to *Caenorhabditis* and *Pristionchus* and represents a clade IV nematode according to the phylogeny of Blaxter et al (1998), whereas *Caenorhabditis*, *Pristionchus* and the hookworm *Ancylostoma* are all clade V nematodes (Fig. 1C) [32,33]. *S. papillosus* has a complex life history in which parthenogenetic females of *S. papillosus* in the small intestine of the host produce eggs that are released with the feces (Fig. 1B). These eggs can either become infective female larvae (Fig. 1D) developing into parasitic females or become free-living males and females, which produce offspring that become parasites and are all females [41,42].

To test if DAs control infective larvae formation of *S. papillosus*, we collected young larvae from feces of a rabbit infected with parasitic females. When we grew these larvae on agar plates containing Δ4-DA or Δ7-DA, we observed a complete suppression of the formation of infective larvae by Δ7-DA ($p<0.01$) (Fig. 3B). In contrast, the same concentration of Δ4-DA did not show any significant effect ($p=0.90$). These results indicate that Δ7-DA can completely inhibit the development of parasitic females from feces-derived larvae under laboratory conditions.

Next we asked if the progeny of free-living adults, all of which usually form infective larvae, could be prevented from developing into infective larvae. We found that administration of Δ7-DA completely prevented infective larvae formation ($p<0.01$) (Fig. 3C). Instead, the Δ7-DA treated animals developed into individuals with a rhabdiform esophagus characteristic of free-living animals, and a mid-body vulva characteristic of adult females (Fig. 1E-G). No males were found in the Δ7-DA treated progeny of free-living adults. However female adults obtained by the Δ7-DA treatment could mate with the male progeny of parasitic females, and laid eggs that again developed to a free-living adult upon treatment with Δ7-DA (data not shown). These results suggest that Δ7-DA negatively regulates the formation of infective larvae in *S. papillosus* and that exogenous administration of Δ7-DA redirects the development to an additional free-living life cycle. Consistently, we could amplify cDNA sequences encoding putative *Spa-daf-12* from the transcripts of young progeny of parasitic and free-living adults by RT-PCR (Figs. S4B,C; 3D).

The functional conservation of the small molecule ligand Δ7-DA has several evolutionary and practical implications. First, DA-DAF-12 represents an endocrine signaling module of a kind that is a common feature in the regulation of adaptive phenotypic plasticity [43,44]. Endocrine signaling was suggested as a useful mechanism for controlling phenotypic plasticity because

it allows organisms to modulate gene expression in response to environmental fluctuation in a fast and systemic manner. In addition, susceptibility of endocrine systems to both environmental and genetic perturbation may produce phenotypic variations, facilitating evolutionary change. While many studies suggested the conservation of developmental regulation by steroid hormones within insects and within vertebrates [44], no such conserved mechanism has been known to exist in nematodes. This study provides evidence for a conserved steroid signaling in nematodes and builds a platform on which to study the evolution of life histories and its genetic and environmental regulation.

Second, the similar effect of Δ7-DA on *C. elegans* [21], *P. pacificus* and *S. papillosus* supports a common origin of dauer and infective larvae in these species and leads to hypotheses on the evolution of parasitism. Nematode parasitism has evolved multiple times from free-living ancestors [33]. Parasitic nematodes often infect their hosts as specialized infective larvae and their morphological similarities to dauer larvae led to the suggestion that infective larvae might have evolved from dauer larvae of free-living species [1-3]. The conservation of Δ7-DA function suggests that the dauer and infective larvae indeed share a common regulatory basis for cell fate specification, and is consistent with the notion that infective larvae evolved from dauer larvae. The evolution of the parasitic life cycle in *S. papillosus* involved the diversification of the post-dauer development from the post-L3 development to produce highly specialized parasitic and free-living adults, respectively (Fig. 1B). We hypothesize that the gradual evolution of parasitism has been facilitated by phenotypic plasticity [44] enabling the retention of a bacteria-feeding free-living life cycle while already shaping an alternative feeding strategy. Many other parasitic nematodes infect their hosts as dauer-like arrested larvae [1]. In most cases, development to such infective larvae is obligatory. Although the regulatory mechanisms and origin of infective larvae in these species need to be verified individually, specialization of post-dauer development to produce parasitic morphs and the subsequent loss of ancestral free-living life cycle, could serve as a useful working hypothesis for the evolution of parasitism. Finally our data suggest that a DAF-12 ligand with a 3-keto-5α-7-ene (Δ7-DA) rather than a 3-keto-4-ene (Δ4-DA) ring structure [21] might be more strongly conserved. The deep conservation of this small molecule ligand has obvious pharmacological implications for the prevention of problems caused by nematodes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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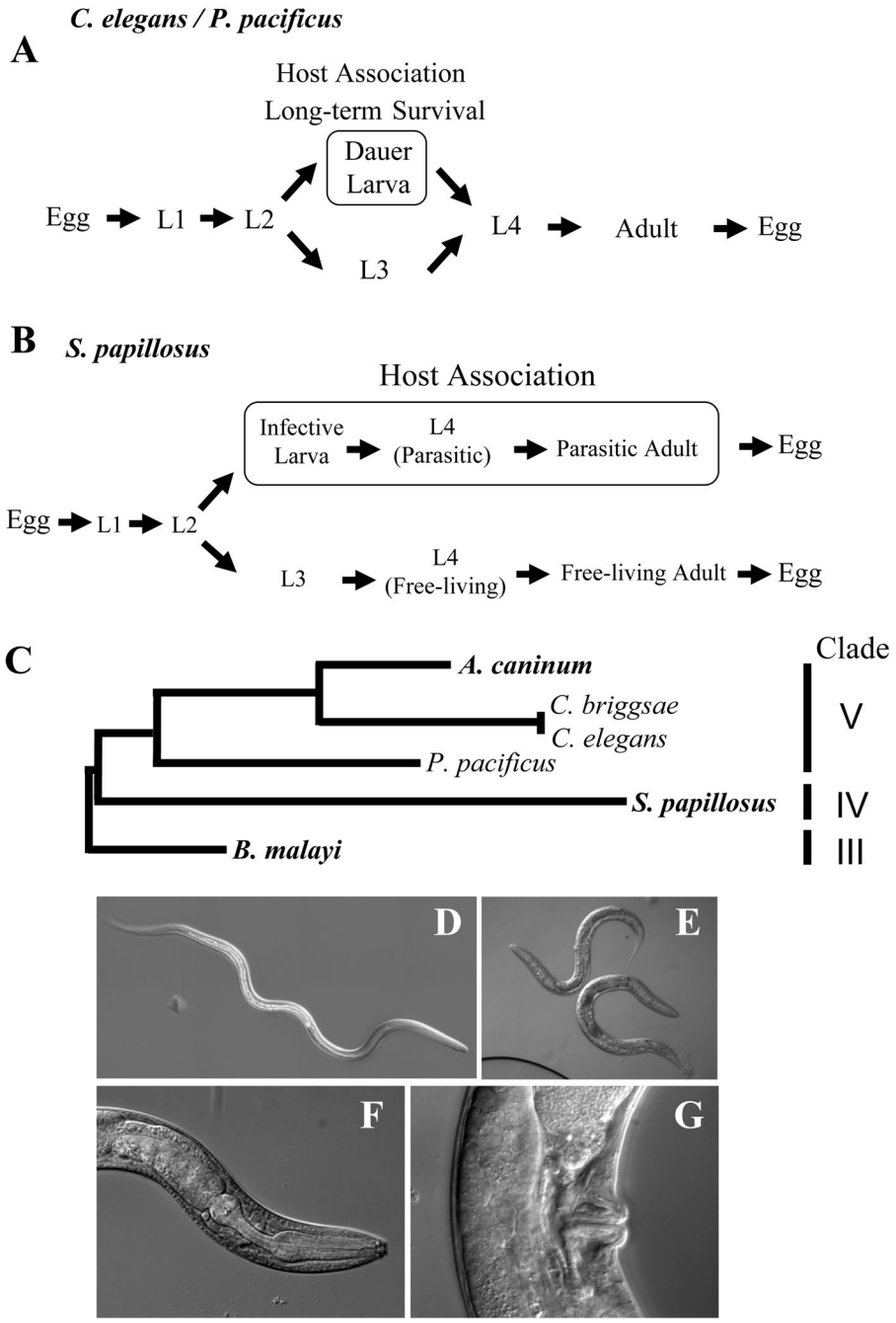

Fig. 1.

(A) Life cycles of the free-living nematodes *C. elegans* and *P. pacificus*. Under favorable conditions, animals go through direct development (L1-L4). In the laboratory, one cycle takes as little as 3-4 days (20° C). Under unfavorable conditions, such as high temperature, high population density or starvation, animals can go into the arrested dauer stage. Note that the importance of the dauer larvae for host association is best known for *P. pacificus* and its beetle association. **(B)** A simplified scheme for the life cycle of the vertebrate parasite *Strongyloides papillosus*. Note that not only the third larval stage, but also the fourth larval stage and the adult stage are specialized for each life style. For a more precise representation of *S. papillosus* life cycle, see Figure S2. **(C)** Phylogenetic relationship of the three species used in

this study (*P. pacificus*, *C. elegans* and *S. papillosus*), with three other taxa *C. briggsae*, the hookworm *A. caninum*, and *B. malayi*. Parasitic species are indicated in bold. **(D)** Overall morphology of a *S. papillosus* infective larva. **E-G** Phenotype of Δ7-DA-treated progeny *S. papillosus* that show morphological characteristics of free-living adults. **(E)** Overall morphology of free-living adults. **(F)** Rhabdiform esophagus with a grinder in the pharynx, which is only known from free-living adults but not parasitic females. **(G)** Vulva opening in the mid-body region, a feature that is also only known from free-living females.

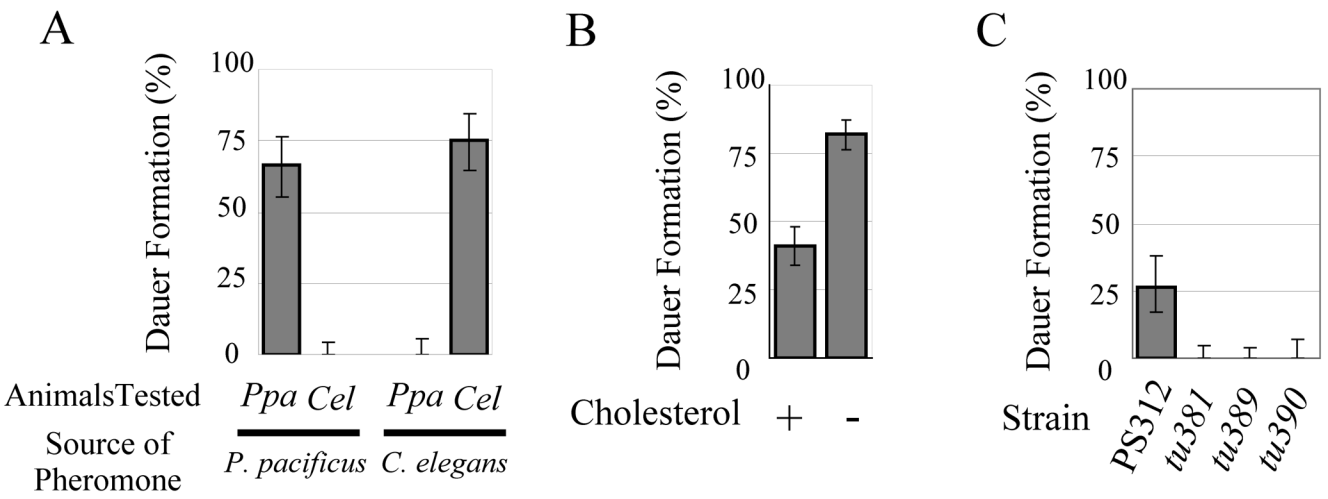

Fig. 2.

Dauer formation assays in *P. pacificus* wild type and mutant animals. **(A)** Dauer formation of *P. pacificus* PS312 (*Ppa*) and *C. elegans* N2 (*Cel*) on agar plates containing either *P. pacificus* or *C. elegans* pheromone. **(B)** Cholesterol restriction enhances dauer formation in *P. pacificus* PS312. Dauer formation was tested on pheromone plates (as in B) in the presence (5μg/ml) or absence of added cholesterol. **(C)** Daf-d phenotypes of the three *Ppa-daf-12* alleles, *tu381*, *tu389*, and *tu390*. Dauer formation of wild type (PS312) and mutant animals was tested on agar plates containing the same amount of pheromone extract. Error bars denote 95% confidence intervals. Note that the experiments described in Figures 2 A, B and C, were performed with different batches and different amounts of pheromone. See Table S1 for number of animals tested.

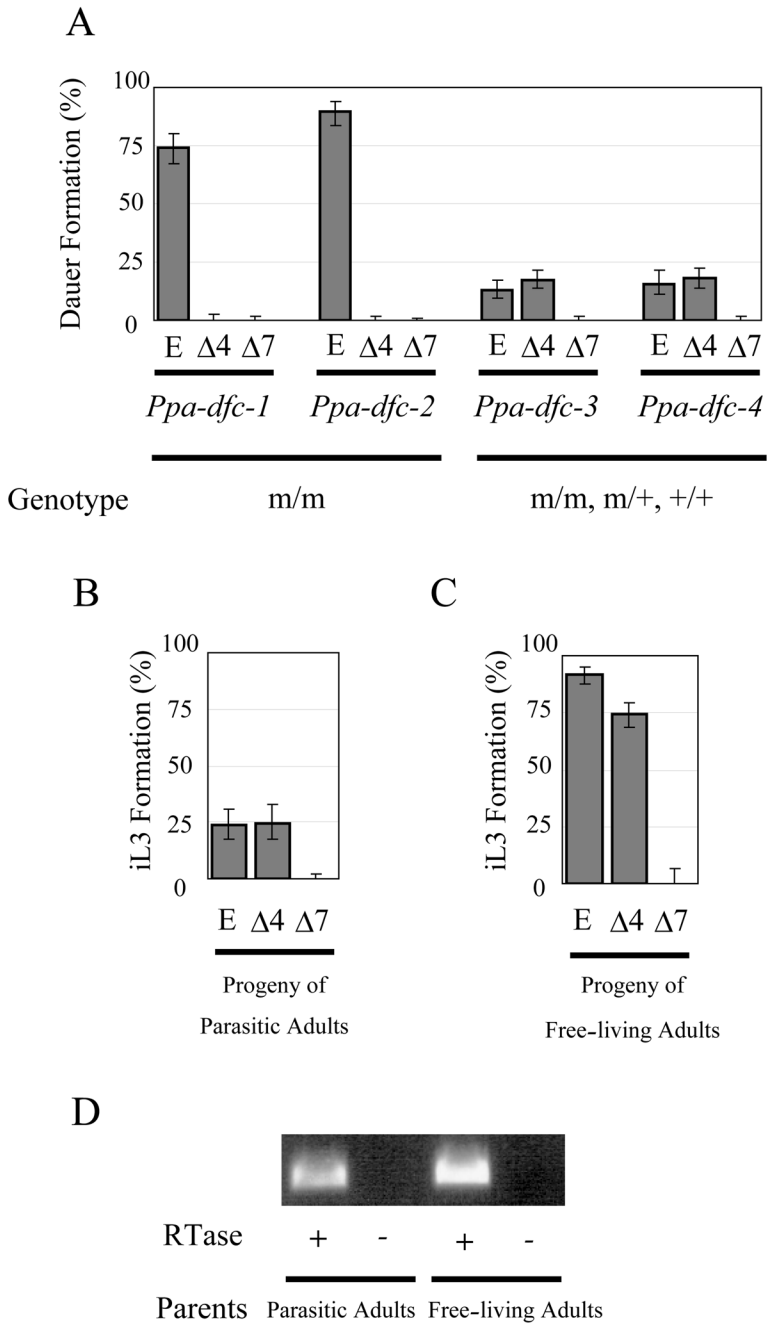

Fig. 3.

Dauer formation assays in *P. pacificus* (A) and infective larvae formation assay in *S. papillosus* (B, C). **(A)** Effect of DAs on dauer formation on four *P. pacificus* Daf-c strains. Mutant animals were grown in the presence of either ethanol (E; control), Δ4-DA (Δ4; 250nM), or Δ7-DA (Δ7; 250nM). For two fully penetrant mutants *Ppa-dfc-3(tu393)* and *Ppa-dfc-4 (tu394)*, mixed populations of homozygous, heterozygous and wild type eggs, which are F1 and F2 progeny of single heterozygous hermaphrodites, were tested. Therefore less than 25% of animals are expected to be mutant. *Ppa-dfc-1(tu391)* and *Ppa-dfc-2(tu392)* were tested as homozygous animals. **(B)** Infective third stage larvae (iL3) formation of the progeny of *S. papillosus* parasitic females in the presence of ethanol (E; control), Δ4-DA (Δ4; 250nM), or

Δ7-DA (Δ7; 250nM). **(C)** iL3 formation of the progeny of *S. papillosus* free-living adults in the presence of ethanol (E; control), Δ4-DA (Δ4; 250nM), or Δ7-DA (Δ7; 250nM). Note that all the non-iL3s in the plates with ethanol and Δ4-DA were small larvae that were presumably younger than the third stage arrested larvae. The majority of non-iL3s in the plate with Δ7-DA were females as shown in Fig. 1E. Error bars denote 95% confidence intervals. See Table S1 for number of animals tested. **(D)** RT-PCR amplification of a partial cDNA of *Spa-daf-12*. RNA was prepared from young progeny of parasitic and free-living adults. Reverse transcriptase (RTase) was omitted in the negative control. The identity of the amplified fragments was verified by DNA sequencing (data not shown)