The cGMP Signaling Pathway Affects Feeding Behavior in the Necromenic Nematode *Pristionchus pacificus*

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Abstract

**Background:** The genetic tractability and the species-specific association with beetles make the nematode *Pristionchus pacificus* an exciting emerging model organism for comparative studies in development and behavior. *P. pacificus* differs from *Caenorhabditis elegans* (a bacterial feeder) by its buccal teeth and the lack of pharyngeal grinders, but almost nothing is known about which genes coordinate *P. pacificus* feeding behaviors, such as pharyngeal pumping rate, locomotion, and fat storage.

**Methodology/Principal Findings:** We analyzed *P. pacificus* pharyngeal pumping rate and locomotion behavior on and off food, as well as on different species of bacteria (*Escherichia coli*, *Bacillus subtilis*, and *Caulobacter crescentus*). We found that the cGMP-dependent protein kinase G (PKG) Ppa-EGL-4 in *P. pacificus* plays an important role in regulating the pumping rate, mouth form dimorphism, the duration of forward locomotion, and the amount of fat stored in intestine. In addition, Ppa-EGL-4 interacts with Ppa-OBI-1, a recently identified protein involved in chemosensation, to influence feeding and locomotion behavior. We also found that *C. crescentus* NA1000 increased pharyngeal pumping as well as fat storage in *P. pacificus*.

**Conclusions:** The PKG EGL-4 has conserved functions in regulating feeding behavior in both *C. elegans* and *P. pacificus* nematodes. The Ppa-EGL-4 also has been co-opted during evolution to regulate *P. pacificus* mouth form dimorphism that indirectly affect pharyngeal pumping rate. Specifically, the lack of Ppa-EGL-4 function increases pharyngeal pumping, time spent in forward locomotion, and fat storage, in part as a result of higher food intake. Ppa-OBI-1 functions upstream or parallel to Ppa-EGL-4. The beetle-associated omnivorous *P. pacificus* respond differently to changes in food state and food quality compared to the exclusively bacteriovorous *C. elegans*.

Introduction

Evolutionary changes in development and behavior take place in the context of ecology. In turn, what and how organisms eat are the most salient aspects of their ecology readily observable under laboratory conditions. Because of its exclusively bacteriovorous diet, relatively simple neuronal architecture, and genetic tractability, the nematode *Caenorhabditis elegans* is an attractive model for the study of how gene expression impinges upon feeding behavior, and how food availability and quality can interact with genotypes [1,2]. In *C. elegans*, as well as in other free-living nematodes, the most prominent organ involved in feeding is the pharynx. Food intake begins at the buccal cavity and is pumped into two pharyngeal bulbs composed of the anterior bulb (corpus), the isthmus, and the posterior bulb (terminal bulb). Contraction of the pharyngeal muscle groups (pumping) followed by posteriorly sweeping relaxation of the muscles in the isthmus (peristalsis) result in food ingestion in *C. elegans* [3].

*P. pacificus* is a necromenic nematode specifically associated with several species of phytophagous beetles around the globe, in particular the Oriental Beetle *Exomala orientalis* in Japan and Northeastern US, as well as the *Cyclocephala* masked chafers in Southern California (RL Hong, unpublished results) [4–6]. Free-living *P. pacificus* populations can also be found in the soil and maintained on strict bacterial diets in the laboratory. *P. pacificus* belongs to the Diplogasteridae family of nematodes whose common ancestor with the Rhabditidae family diverged approximately 250–420 million years ago [7]. Unlike the Rhabditid *C. elegans*, Diplogastrids lack pharyngeal grinders in the terminal bulb (posterior pharynx) that help to breakup bacteria but instead have a larger buccal cavity anterior to the pharynx that contain two chitinous teeth. *P. pacificus*, in particular, have phenotypic dimorphism consisting of two mouth forms, one with a narrower buccal cavity called the stenostomatous form, and another with a broader buccal cavity known as the eurystomatous form [8–10]. The evolution of teeth in *Pristionchus* nematodes may be advantageous for the necromenic lifestyle in which *Pristionchus* can feed on various food sources such as bacteria, fungi, and other
nematodes. All *Pristionchus* species however, can sustain itself on an *E. coli*-only diet in the laboratory.

Despite such divergent feeding physiology and natural ecology, we currently have sparse knowledge of *P. pacificus* feeding behavior. A recent study showed that *P. pacificus* decrease forward bending frequency from on to off food state whereas *C. elegans* increase forward bending frequency when transferred from on to off food [11]. Other free-living nematodes such as *Oscheius myriophila*, *Rhabditia*, and *Pellioditis typica* display similar reactions as *C. elegans* to changes in food state [11]. Only *Panagrellus redivivus* displayed the same feeding behavior as *P. pacificus*. Changes in foraging behavior by speeding up or slowing down locomotion presumably need to be coordinated with changes in food intake. In *C. elegans*, the feeding rate is measured by the rate of pharyngeal pumping—pumping is higher on food than in the absence of food [12]. Thus *P. pacificus* responds to a lack of food by slowing down locomotion, whereas *C. elegans* increase locomotion but reduce pharyngeal pumping when food is removed. This study will address how *P. pacificus* adjust food pumping rate in response to food availability and food quality.

In *C. elegans*, a pleiotropic gene known to integrate environmental perception, behavior, and growth is *Cel-egl-4*. Cel-EGL-4 is a highly conserved cGMP dependent protein kinase G (PKG) important for a variety of food-seeking behaviors, such as pharyngeal pumping, chemosensation, and locomotion [13–16]. PKGs in other invertebrates such as the fruit fly *Drosophila* and the honeybee *Apis mellifera* also play prominent roles in regulating foraging and a polymorphism in PKG is maintained in wild *Drosophila* populations [17,18]. Past studies in *P. pacificus* demonstrated that differences in *Ppa-egl-4* expression level is involved in the natural polymorphism for an insect sex pheromone attraction between two *P. pacificus* strains [16]. In this study, we found that the *Ppa-egl-4(tu374)* null allele animals are predominantly stenostomatous in mouth form, compared to the ~2:1 stenostomatous:eurystomatous ratio found in the wild-type PS312 population [10]. Since EGL-4 function is conserved across phyla and can generate phenotypic changes both at the macroevolutionary level (from insects to nematodes), as well as at the microevolutionary level (within *Drosophila* and *P. pacificus* populations), we sought to address in this study the hypothesis that EGL-4 can also be responsible for coordinating genetic changes in feeding behavior at an intermediate evolutionary scale by comparing the feeding behaviors of *P. pacificus* and *C. elegans* nematodes.

**Results**

**P. pacificus** Pharyngeal Pumping Rate Changes in Response to Food Availability and Food Type

Both *P. pacificus* and *C. elegans* live solely on *E. coli* OP50 under normal laboratory conditions. To investigate whether food types can alter pumping rate, we cultured *P. pacificus* PS312 for more than six generations on two other bacterial species found in soil—the gram-negative *Caulobacter crescentus* NA1000 found in freshwater and neutral soil [20] and the gram-positive *Bacillus subtilis* PY79 [21]. Wild-type PS312 cultured on *E. coli* OP50 showed ~25% increased pharyngeal pumping rate (pumps per minute of the corpus, ppm) when removed from food for at least 5 minutes. Similarly, PS312 cultured on *C. crescentus* and *B. subtilis* showed increased ppm when removed from food by ~23% and ~26%, respectively (Fig. 1B). However, we were surprised to find the magnitude of increase in pumping to be the same, although these 3 bacterial species differ in size (*C. crescentus* = *E. coli* < *B. subtilis*) and growth rate (*C. crescentus* < *B. subtilis* < *E. coli*) [3,22,23]. Interestingly, *P. pacificus* pumps more rapidly both on and off *C. crescentus* strains.
cGMP Signaling in Nematode Pristionchus pacificus

The cGMP Signaling Pathway is Important for Controlling Pharyngeal Pumping Rate in P. pacificus

P. pacificus is a free-living nematode capable of eating non-bacterial food such as fungi and other nematodes [9,10]. Previous comparative studies highlighted that one major difference between P. pacificus and C. elegans foraging behavior is the decrease in locomotion from on to off food state [11]. Hence, we examined to see if there are other differences in feeding behavior in P. pacificus, such as food intake rate. In C. elegans wild-type N2, the pharyngeal pumping rate is measured by counting the pumping movements of the grinder in the terminal bulb of the pharynx, and pumping decreases initially when transferred from a well-fed state on food (e.g. E. coli OP50) to an off food state without any bacteria (<1 hour) [12] Because P. pacificus do not pump in the terminal bulb but instead pump only in the corpus (anterior bulb), we measured pumping rate by visually counting the contractions of the corpus. We found that, unlike C. elegans N2, P. pacificus wild-type PS312 showed increased pumping rate when off food compared to on food, although both rates are much lower than the pumping rate of C. elegans (>200 ppm).

To determine which genes affect pumping rate, we first measured the pumping rate of the Ppa-egl-4(tu404) loss-of-function null allele [16]. P. pacificus EGL-4 is the ortholog of the C. elegans cGMP dependent protein kinase G (PKG) known to regulate pharyngeal pumping, chemosensation, and locomotion [13–15,23,38]. The Cel-egl-4 loss-of-function mutants lack feeding quiescence after fasting and pumps faster than wild-type N2 on food (pers. comm. van Buskirk) [3]. We found that the loss of Ppa-egl-4 function also greatly increased pumping off food compared to wild-type PS312, although pumping on food remained the same between wild-type and Ppa-egl-4. The Ppa-egl-4 locus has also been shown to be involved in the natural polymorphism of chemotraction towards the lepidopteran sex pheromone E-1-tetradecylacetate (E-TDA) [16]. P. pacificus Washington (PS1843) is strongly attracted to E-TDA whereas the California (PS312) reference strain is completely insensitive to E-TDA. However, a brief one-hour soaking treatment of PS312 with the stable, cell permeable 8-bromo-cGMP can increase Ppa-egl-4 transcript level and induce PS312 chemotraction to E-TDA [16]. The same exogenous cGMP treatment can also induce PS312 chemoattraction to the sex pheromone of the Oriental Beetle, Z7,7-tetradece-2-one (Z-TDO), but this cGMP-dependent ZTDO attraction does not wholly depend on Ppa-egl-4 [16].

Next, in an effort to find other factors that mediate the exogenous cGMP-dependent attraction to Z-TDO in P. pacificus PS312, we isolated an Oriental Beetle pheromoneInsensitive allele, Ppa-obi-1(tu404), that no longer chemotax to Z-TDO after cGMP treatment (RL Hong, unpublished data). We wondered if the loss of Ppa-obi-1, in addition to altering chemosensation, could also affect feeding behavior. We found that Ppa-obi-1 animals had a lower pumping rate on E. coli compared to wild-type, an effect that is suppressed by the loss of EGL-4 function. However, Ppa-obi-1, Ppa-egl-4, and Ppa-egl-4; Ppa-obi-1 double mutants displayed a significantly higher pumping rate off food than wild-type PS312, although this increase is more variable in Ppa-obi-1 animals. This result suggests that Ppa-OB1 promotes pumping on food but negatively regulates Ppa-egl-4 to control pumping rate off food. Thus, the loss of Ppa-obi-1 resulted in decreased pumping rate on food while the loss of Ppa-egl-4 and/or Ppa-obi-1 resulted in increased pumping rate off food (Fig. 2A). The mutant phenotypes also imply that pharyngeal pumping on and off food are regulated differently by the cGMP pathway.

Since P. pacificus cultured on C. crescentus displayed increased pumping on and off food, we asked if Ppa-egl-4, which regulates pumping rate both on and off E. coli, also regulates this process on C. crescentus. We found that both Ppa-egl-4 and Ppa-egl-4; Ppa-obi-1 double mutants on C. crescentus NA1000 showed increased pumping on food, but not off food (Fig. 2B). The loss of Ppa-obi-1 did not affect pumping rate on C. crescentus. In contrast, Ppa-obi-1 off food had decreased pumping. The reduction in Ppa-egl-4 or Ppa-obi-1 function also reduced the magnitude of pumping increase from on to off food animals grown at 15°C. However, Ppa-egl-4 on C. crescentus displayed an additional 3% increase in pumping rate compared to wild-type. The difference in pumping rate of both wild-type and Ppa-egl-4 animals, however, off C. crescentus is due in part to the inability of the muscles to pump faster at 20°C, the degree of difference on and off C. crescentus in Ppa-egl-4 is fixed at 20°C, or off E. coli. This finding

Pharyngeal pumping rate of food states on the smallest strain (wild-type NA1000) is significant. (Two-tailed t-test, P < 0.001). doi:10.1371/journal.pone.0034464.g001
also suggests that Ppa-EGL-4 has two roles: Ppa-EGL-4 controls pumping rate off food regardless of food type but controls pumping rate in a food-specific manner, such as on C. crescentus, but not on E. coli. In contrast, Ppa-obi-1 controls pumping rate both on and off food for E. coli, but only off food for C. crescentus.

Ppa-EGL-4 Affects Mouth Form Dimorphism and Pumping Rate in P. pacificus

Unlike C. elegans, distinct genetically homogeneous P. pacificus populations are each composed of two distinct subpopulations with different mouth forms [8–10]. The ratio of stenostomatous to eurystomatous mouth forms in the wild-type PS312 population (51±17% stenostomatous) can be altered by passage through the dauer larvae stage (100% stenostomatous) [10]. Interestingly, we found that Ppa-egl-4(tu374) is a genetic mutant that distorts the mouth form ratio towards a high percentage of the stenostomatous morph (87±11%). Because the stenostomatous mouth form has a narrower, longer buccal cavity, more stenostomatous animals in the Ppa-egl-4 population contribute to the increase in pharyngeal pumping rate in Ppa-egl-4 animals.

To determine if the loss of Ppa-egl-4 affected directly the pumping rate via pharyngeal muscles or indirectly via distortions in mouth form ratio in the population, we sought to measure the pumping rate of a population composed of only a single mouth morph. To do this, we measured dauer passaged wild-type and Ppa-egl-4 animals cultured on OP50 because they are nearly 100% stenostomatous (Fig 3). We found that the predominantly stenostomatous wild-type population pumped faster than the not-dauer passaged animals, although still not as fast as the Ppa-egl-4 mutants, regardless of dauer passage. Therefore, the increased pumping rate in the Ppa-egl-4 mutants is due both to a direct physiological effect on pharyngeal neurons as well as an indirect developmental effect of increasing the likelihood of stenostomatous mouth formation.

Figure 2. Pharyngeal pumping rate of P. pacificus mutants on/off OP50 E. coli and NA1000 C. crescentus (mean±SEM). (A) Pumping on/off OP50 E. coli. Wild-type PS312 (n = 25–36), Ppa-egl-4(tu374) (n = 17–32), Ppa-obi-1(tu404) (n = 13–20), Ppa-egl-4; Ppa-obi-1 (n = 15). The difference in pumping between on/off food states for each nematode strain is significant (not indicated; two tailed t-test, P<0.001). Pumping rate on food is significantly slower in Ppa-obi-1(tu404) compared to the other 3 genotypes, whereas pumping rate off food is significantly faster in all mutants compared to wild-type PS312 (Dunnett’s Multiple comparisons test, P<0.01). (B) Pumping on/off NA1000 C. crescentus. PS312 (n = 35–38), Ppa-egl-4 (n = 16–18), Ppa-obi-1(tu404) (n = 14–18), Ppa-egl-4; Ppa-obi-1 (n = 14–18). The difference between on/off food states for each nematode strain is significant (two tailed t-test, P<0.001). Pumping rate off food is significantly slower in Ppa-obi-1(tu404) compared to the other 3 genotypes, whereas Ppa-egl-4 and Ppa-egl-4; Ppa-obi-1 pump faster than wild-type (Dunnett’s Multiple comparisons test, P<0.05, *0.001). (C) Pumping on/off on C. crescentus NA1000 at 15°C. PS312 (n = 26–32), Ppa-egl-4 (n = 22–24). The rate difference between on/off food states for Ppa-egl-4 is significant (two tailed t-test, P<0.001). Ppa-egl-4 pumps faster than wild-type on C. crescentus (Dunnett’s Multiple comparisons test, P<0.001). doi:10.1371/journal.pone.0034464.g002

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To determine if the loss of Ppa-egl-4 affected directly the pumping rate via pharyngeal muscles or indirectly via distortions in mouth form ratio in the population, we sought to measure the pumping rate of a population composed of only a single mouth morph. To do this, we measured dauer passaged wild-type and Ppa-egl-4 animals cultured on OP50 because they are nearly 100% stenostomatous (Fig 3). We found that the predominantly stenostomatous wild-type population pumped faster than the not-dauer passaged animals, although still not as fast as the Ppa-egl-4 mutants, regardless of dauer passage. Therefore, the increased pumping rate in the Ppa-egl-4 mutants is due both to a direct physiological effect on pharyngeal neurons as well as an indirect developmental effect of increasing the likelihood of stenostomatous mouth formation.

Ppa-egl-4 Mutants Show Increased Roaming and Forward Velocity

Nematodes on solid media can be observed as moving forwards, backwards, or not at all (stopped for at least 4 seconds). Unlike C. elegans, which increases locomotion (body bends/minute) when transferred from on to off food, P. pacificus decreases locomotion when deprived of food from a well-fed state [11]. Because the pharynx pumps constantly, we wished to determine if the increase in pumping rate of Ppa-egl-4 off food is coupled with alterations in foraging behavior. The PKG in other invertebrates such as Drosophila and honey bee also play a key role in food-seeking behavior [17,18]. In C. elegans, Cel-egl-4 loss-of-function mutants...
show increased roaming behavior on food compared to wild-type, as exhibited by a more time spent on forward locomotion [14,26], while Cel-egl-4 gain-of-function mutants displayed increase in dwelling, as signified by more stops and reversals [25]. We compared the foraging behaviors of the various P. pacificus feeding mutants in the absence of food (E. coli OP50). The loss-of-function Ppa-egl-4(tu374) null animals spent more percentage of time off food moving forward (P<0.05, Fig. 4A). In contrast, the Ppa-obi-1 mutant showed decreased reverse durations compared to wild-type (P<0.01), although the durations moving forward were not significantly different than wild-type. Furthermore, Ppa-egl-4; Ppa-obi-1 animals spent more of their time not moving compared to the wild-type or single mutants (P<0.05), and also differs in duration of forward and reverse movements compared to wild-type (P<0.01). Thus, the loss-of-function Ppa-egl-4 mutants in P. pacificus, as in the case of C. elegans, displayed an increased proportion of forward movements, while the Ppa-obi-1 mutation completely reversed the Ppa-egl-4 roaming phenotype. However, the Ppa-egl-4; Ppa-obi-1 double mutant animals displayed forward and stopped durations not observed in the single mutants while the reduced reversal duration of Ppa-obi-1 appears to be epistatic to Ppa-egl-4.

In addition to differences in the percentage of time spent on certain movements, we examined whether P. pacificus and C. elegans differ fundamentally in speed and reaction to change in food state by measuring mean forward velocity on and off food. We found that not only is P. pacificus significantly slower than C. elegans by 4–5 fold both on and off food, P. pacificus also does not display a dramatic increase in velocity when food is removed from well-fed individuals as is the case in C. elegans (Fig. 4B). Furthermore, we found differences between wild-type P. pacificus and mutants in the cGMP pathway. Specifically, although Ppa-egl-4 mutant animals

**Figure 3. Mouth form affect pharyngeal pumping rate off OP50 E. coli.** The proportion of young adult hermaphrodites with stenostomatous mouth form changes in the population with dauer passage (d) (percentage of stenostomatous indicated inside the bars). The pumping rate of stenostomatous dauer passed wild-type is intermediate between not-dauer passed wild-type and the mostly stenostomatous Ppa-egl-4 mutants, regardless of dauer passage (Dunnett’s Multiple comparisons test, P<**0.01,** ***0.001). doi:10.1371/journal.pone.0034464.g003

**Figure 4. Locomotion of P. pacificus mutants (mean±SEM).** (A) Proportion of durations P. pacificus mutants spent off food (E. coli OP50) moving forward, moving backwards, and not moving. PS312 (n = 7), Ppa-egl-4 (n = 20), Ppa-obi-1 (n = 18), Ppa-egl-4/Ppa-obi-1 (n = 9). The percentage time spent is significantly different between wild-type and the mutant strains (Dunnett’s Multiple Comparisons Test *P<0.05). (B) Forward velocities of C. elegans N2 and P. pacificus strains measured on or off food. Velocity is in millimeters per second cultured on E. coli OP50. C. elegans wild-type N2 (n = 31–94), P. pacificus wild-type PS312...
spent more time moving forward, they do not move faster on or off food compared to wild-type. Ppa-obi-1 however, is slower than wild-type on food. In contrast, the Ppa-egl-4; Ppa-obi-1 double mutants have significantly reduced forward velocity off food compared to the wild-type or Ppa-egl-4 mutants alone. We speculate that Ppa-OB1 acts upstream of Ppa-egl-4 to positively regulate forward velocity in the absence of food, although in a more complex manner than for pharyngeal pumping. Interestingly, the Ppa-obi-1 mutant exhibited a similar degree of strong increase in velocity from on to off food (40% faster off food) observed in the C. elegans wild-type (30% faster off food).

We found equally profound differences between P. pacificus and C. elegans when we focused on the reversal frequency. Whereas C. elegans wild-type moved ~5x faster forward, P. pacificus wild-type reversed almost 4x more frequently than C. elegans (Fig 4C). Given its slow forward velocity, P. pacificus may instead increase reversal frequency to enhance coverage of the local area rather than increase its foraging range when confronted with possible starvation. The reversal frequency of Ppa-egl-4 and Ppa-obi-1 were less on food than off food in contrast with wild-type and Ppa-egl-4; Ppa-obi-1 double mutants. In the case of Ppa-egl-4; Ppa-obi-1 double mutants however, reversal frequency was equally low on and off food. The decreases in reversal frequency coincided with increases in pumping rate off food in Ppa-egl-4; Ppa-obi-1, and Ppa-egl-4; Ppa-obi-1 double mutants. This result suggests that when P. pacificus lacks food, Ppa-egl-4 functions to coordinate foraging patterns by regulating the proportion of time moving forward, reversal frequency, and the rate of pharyngeal pumping.

**C. crescentus Increases P. pacificus Fat Content**

A previous study in C. elegans by Shionda and Avery (2006) demonstrated that the presence of high quality food correlated with changes in locomotion behavior [40]. We hypothesized that the observed difference in pharyngeal pumping behavior due to differences in food or genotype would also influence the nutritional status of the nematodes. Wild-type P. pacificus pumped food more rapidly on and off C. crescentus NA1000 than on and off E. coli OP50. We therefore asked if the increase in pumping on C. crescentus has consequences in fat accumulation because it is more nutritious. We utilized the Nile Red dye to detect triacylglyceride (TAG) content in the intestines of fixed animals [27]. The size of stained lipid bodies in the anterior intestine of C. elegans N2 and P. pacificus PS312 were similar in size when fed on E. coli OP50, but increased dramatically when P. pacificus strains were cultured on C. crescentus (Fig. 5). This supports the notion that the faster pumping on C. crescentus may be driven by preference for bacteria that provide higher fat content, rather than a reaction to lower density of food per pump. Interestingly, C. elegans cultured on C. crescentus did not show a significant increase in fat storage compared to those cultured on OP50 and thus may reflect a very different metabolism from P. pacificus (Fig. 6). Next, we investigated whether the higher pumping rate in Ppa-egl-4 (tag374) on C. crescentus would result in even higher fat accumulation. To our surprise, the fat storage level was unchanged compared to the wild-type on C. crescentus, but the fat level in Ppa-egl-4 cultured on OP50 was found to be higher than in wild-type PS312 (P<0.05). This result is in contrast to the previous finding that a Cel-egl-4 gain-of-function allele did not accumulate more fat using the same Nile Red staining method [27]. Unlike the wild-type, Ppa-obi-1 did not accumulate more fat when cultured on C. crescentus (P<0.001, Tukey-Kramer). Thus, C. elegans N2 did not seem to accumulate fat differently on E. coli and C. crescentus, whereas P. pacificus PS312 accumulated almost 3x more fat on C. crescentus compared to on E. coli. The higher fat accumulation also correlated with the faster pharyngeal pumping on C. crescentus compared to on E. coli in wild-type P. pacificus. However, this correlation between pharyngeal pumping rate and fat storage level did not hold for Ppa-egl-4 and Ppa-obi-1 mutants, perhaps due to the distinct but overlapping regulatory circuits for pharyngeal pumping on and off food. Alterations in these two genes can affect the magnitude of this fat storage difference between E. coli and C. crescentus diets. Ppa-egl-4 (tag374) specifically increases the fat level on OP50 compared to wild-type, whereas Ppa-obi-1 reduces the fat level on C. crescentus compared to wild-type.

**Discussion**

*P. pacificus* is a necromenic nematode specifically associated with several species of beetles, whereas *C. elegans* is primarily found in vegetable composts [5,6,19,28]. In the course of evolution, their contrasting ecologies produced stark differences in feeding behavior as evident by their feeding organs. *P. pacificus* lack pharyngeal grinders but have teeth and show predatory behavior towards *C. elegans* and other nematodes [RJ Sommer, personal communication] [10]. The ability of *P. pacificus* to catch and kill moving prey, coupled with their ability to proliferate in the laboratory solely on bacteria, suggest *P. pacificus* has a flexible diet and potentially complex feeding strategies in nature. Our present study interrogates the relationship between *P. pacificus* genotypes and their responses to food states and food types.

**Comparison of EGL-4 Functions**

The PKG EGL-4 has conserved functions in regulating feeding behavior in both *C. elegans* and *P. pacificus*. Specifically, the Ppa-egl-4 mutants increased their rate of pharyngeal pumping, along with increased duration roaming off food through increased proportion of time moving forward. Interestingly, the Ppa-egl-4 mutants did not pump faster on OP50 but only off of OP50. One interpretation is that animals lacking Ppa-egl-4 are more sensitive to the removal of food. This sensitivity may also depend on food metabolism, as shown by the increase in fat storage of Ppa-egl-4 grown on OP50. *P. pacificus* pumped faster when food is more nutritious (on *C. crescentus*), or when the higher fat storage of the Ppa-egl-4 mutant animals demands even more food intake from less nutritious food (on *E. coli*). PS312 cultured on *C. crescentus* also seemed to induce more fat accumulation as demonstrated by the animals’ increased pharyngeal pumping and fat storage. However, the nutritional content of *C. crescentus* NA1000 may be significantly different from *E. coli* OP50. Although Ppa-egl-4 on *C. crescentus* also showed increased pumping on food, Ppa-egl-4 did not show further
increase in fat content, perhaps because the larger size of fat droplets in wild-type *P. pacificus* cultured on *C. crescentus* is already close to the physiological limit. We observed that *Ppa-obi-1*, a chemosensory mutant that does not respond to exogenous cGMP, showed *egl-4*-dependent phenotypes in pharyngeal pumping. This suggests that Ppa-obi-1 acts upstream to negatively regulate Ppa-egl-4 in feeding behavior (Fig 7).

In addition to conserved physiological functions, our findings also indicate that Ppa-egl-4 has also been co-opted to regulate the mouth form dimorphism that is found in the *Pristionchus* genus. We found that wild-type PS312 nematodes are 51 ± 17% stenostomatous, while *Ppa-egl-4* (tu374) nematodes are 87 ± 11% stenostomatous. However, it is not clear if *Ppa-egl-4* normally represses stenostomatous development or promotes eurystomatous development. The development of the mouth form is phenotypically plastic within a single genotype and is influenced by environmental signals, such as starvation [10]. Passage through the dauer larvae, another phenotypically plastic trait induced by starvation, greatly increases the proportion of stenostomatous animals in the population. The stenostomatous mouth form is partly characterized by a longer, narrower buccal cavity that can restrict passage of food particles, hence the mostly stenostomatous *Ppa-egl-4* animals may pump faster to compensate for receiving less food per pump. To discriminate between the direct regulation of pharyngeal neurons versus the indirect developmental effect of mouth form dimorphism by Ppa-egl-4, we compared dauer passed wild-type and *Ppa-egl-4* mutants (both ~100% stenostomatous). Our finding that dauer passed wild-type pumps faster than not-dauer passed wild-type (with less stenostomatous) but still slower than *Ppa-egl-4*, regardless of dauer passage, suggests both direct and indirect effect of Ppa-egl-4 on pharyngeal pumping (Figs. 3 and 7). Therefore, Ppa-egl-4 regulates feeding behavior by integrating environmental signals with development and physiology. In the wild, it may be advantageous for starvation induced *P. pacificus* dauer juveniles that recovery on limited food to have more individuals with stenostomatous mouth forms that pump faster when subsequently confronted with low food patches.

The role of PKG in regulating food acquisition behaviors is conserved across large evolutionary distances, suggesting the high connectivity of PKG to various cellular signals, from nematodes to humans [29,30]. However, how PKG functions diverge with respect to specific roles in feeding and metabolism has not been investigated at a shorter evolutionary scale, *i.e.* between members of different taxonomic families such as between the Rhabditidae and Diplodogasteridae. Table 1 summarizes and compares the various functions of Ppa-egl-4 in *P. pacificus* and *C. elegans* based on genetic analyses. Interestingly, although PKG regulate locomotion and pharyngeal pumping in both *P. pacificus* and *C. elegans*, these two nematodes species react in opposite manners to food removal. *C. elegans* respond to food removal by moving faster and pumping less, while *P. pacificus* neither change forward velocity nor reversal frequency, and pumps faster when food is gone. One interpretation for these strategies is that *C. elegans* are more “confident” that they will get to a food source soon but refrain
However, the Nile Red staining on worms more accurately reflects fat content [27]. Nile Red staining on live worms and recent work demonstrated that staining on fixed animals is exhibited by EGL-4 [32] or the diverged functions between the two known PKGs in C. elegans and P. pacificus [35], suggesting that P. pacificus cultured on C. crescentus may find it more nutritious. Our future research will seek to address how different strains of C. crescentus may influence pharyngeal pumping rate and metabolism in P. pacificus.

C. crescentus Induces Faster Pumping and Fat Storage

Microfauna studies and transmission electron micrographs strongly suggest that E. coli OP50 is not effectively digested by P. pacificus because intact E. coli cells remain in the intestine [33,34]. This may be because P. pacificus lacks grinders to break up bacteria from maximum pumping until they find food, while P. pacificus are less “confident” that food is nearby so they remain in the same foraging pattern but pump faster to get the most out of the food that remains. Another major difference between the two nematodes is exhibited by EGL-4’s role in controlling body size. Previous studies show that the loss of EGL-4 function increased body size in C. elegans but decreased body size in P. pacificus [16,31]. The loss of EGL-4 function in C. elegans reduced fat content whereas Ppa-egl-4(tu374) mutants showed higher fat content [25]. However, the Nile Red staining on Cel-egl-4(If(f)) has been performed on live worms and recent work demonstrated that staining on fixed worms more accurately reflects fat content [27]. Nile Red staining on fixed gain-of-function allele Cel-egl-4(ad450) specimen did not differ from wild-type N2 in fat storage. The observed phenotypic differences between P. pacificus and C. elegans egl-4 loss-of-function mutants may be due to changes in EGL-4 transcriptional targets [32] or the diverged functions between the two known PKGs in their genomes or a combination of both. Both the C. elegans and the P. pacificus genomes encode another transcribed PKG paralog (WBGene00015650). Therefore the egl-4 null alleles in both nematode species likely represent a reduced rather than a complete lack of PKG activities, and thus additional studies on the distributions of functions between the PKG paralogs in both species are needed.

C. crescentus and P. pacificus are both gram-negative bacteria. However, E. coli has an optimal growth temperature of 37°C commonly found in the intestines of endotherms, while C. crescentus is found in freshwater and sub-terrestrial environments with optimal growth temperatures between 23–28°C [20], conditions much closer to where P. pacificus and C. elegans are found in nature. C. crescentus is a model for understanding prokaryotic development and cell cycle regulation because of its dimorphic life cycle involving motile swimmers with flagellum and sessile cells with adhesive stalks [24]. In particular, phospholipids are the primary fatty acids in the cell membranes of bacteria and do not differ significantly among E. coli strains, whereas triacylglycerides have been found to differ significantly among OP50, HB101, and HT115 strains of E. coli [27]. In addition to cell length, the C. crescentus strains with defects in DNA replication used in this study can also differ in metabolism as well as cell cycle rate (S Murray, personal communication). A recent study also indicated that C. elegans pump faster on non-pathogenic bacteria than on pathogenic bacteria, especially when selected for fitness during experimental evolution [35], suggesting that P. pacificus cultured on C. crescentus may find it more nutritious. Our future research will seek to address how different strains of C. crescentus may influence pharyngeal pumping rate and metabolism in P. pacificus.

Table 1. A comparison of egl-4 mutant phenotypes in C. elegans and P. pacificus.

| egl-4 mutation | body length (mm) | fat storage | pharyngeal pumping | Forward movement | egg holding | odor sensing | odor adaptation |
|----------------|------------------|-------------|---------------------|------------------|-------------|-------------|----------------|
| Cel-egl-4(If(f)) | ↑26,31           | ↓25         | ↑25                 | ↑14              | ↑18         | ↓13         | ↓13            |
| Ppa-egl-4(tu374) | ↓16              | ↑*          | ↑*                  | ↑*               | ↑*          | ↑*          | ↑*             |
| Ppa-egl-4(lf)   | ↑16              | ↑16         | ↑16                 | ↑16              | ↑16         | ↑16         | ↑16            |

In C. elegans, the loss-of-function (If) alleles n479 and k562 have been used in multiple studies; in P. pacificus, only the loss-of-function null allele tu374 have been described. For fat storage measurements, Cel-egl-4(If) and Ppa-egl-4(If) alleles refer to Nile Red staining on fixed animals. *this study; †van Buskirk pers. comm; [13,14,16,25,26,31,38].

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feeding phenotypes, Ppa-OBI-1 acts both upstream as well as in parallel to Ppa-EGL-4 in signaling pathways involving other yet-to-be identified factors. Preliminary studies on Ppa-egl-4 and Ppa-obi-1 show largely non-overlapping expression patterns, suggesting that these two cGMP signaling components intersect functionally but are expressed in distinct cell types (RL. Hong, unpublished results).

**Materials and Methods**

**Nematode and Bacterial Strains**

Nematode strains were cultured between 20–23°C. The wild-type strains of *C. elegans* and *P. pacificus* were N2 Bristol and PS312 California, respectively. *Ppa-egl-4*(tu374) and *Ppa-obi-1*(tu404) were generated in the PS312 background by UV and X-ray mutagenesis and are presumed to be both loss-of-function null mutations (Hong et al 2008 and unpublished results to be described elsewhere). *Ppa-egl-4; Ppa-obi-1* double mutants were obtained by PCR genotyping F2 cross progeny with the long body phenotype of *Ppa-obi-1*(tu404) for the 780 bp *Ppa-egl-4*(tu374) deletion [16]. For bacteria, *Escherichia coli* OP50, *Bacillus subtilis* PV79 and *C. crescentus* NA1000 were the reference strains. *E. coli* and *B. subtilis* cultures were inoculated in LB at 37°C while *C. crescentus* cultures were grown in Peptone Yeast Extract (PYE) at 28°C. *C. crescentus* strains with defects in ctrA required for proper DNA replication resulted in the larger cell sizes of SM921 (longer, ctrAP1?)(S. Murray, personal communication) and LS2195 (longest, ctrA405) [24]. 150 μl of fresh overnight bacteria inoculates were seeded onto 6-cm culture plates containing Nematode Growth Media (NGM). Nematode cultures were acclimated for at least 6 generations on each bacterial strain before being assessed for pumping rate, locomotion, and fat storage.

**Pharyngeal Pumping Rate**

In *C. elegans*, the pharyngeal pumping rate is measured by visually counting the contractions of the “grinder” in the posterior, or terminal pharyngeal bulb. Counts are often observed using video recordings because the pumping rate is too fast to count by eye. Unlike *C. elegans*, *P. pacificus* does not have grinders and the terminal bulb does not pump, but instead pumps only in the corpus (anterior pharynx). The counts in *P. pacificus* are <200 pumps per minute (ppm) and measured by visually counting the contractions of the corpus using a ZEISS LUMAR stereomicroscope at 80x magnification. Worms from well-fed cultures were placed on assay plates containing either a thin lawn of an overnight culture of the same bacteria (on food) or on plates not placed on assay plates containing either a thin lawn of an overnight culture of the same bacteria (off food for 5 minutes). For each animal, we counted pumps for five 15-second intervals, with >2 minutes between intervals, and then multiplied its mean by four to derive the mean pumps per minute.

**Mouth Form Dimorphism**

To obtain populations composed primarily of the stenostomatous mouth form, we picked wild-type PS312 and *Ppa-egl-4*(tu374) dauer juveniles from 2–3 week old starved culture plates onto OP50 seeded lawns and let them resume development for 3 days at ~22°C. The mouth forms of young adults of both not-dauer passaged and dauer passaged populations were analyzed at 630x using Nomarski optics and classified as stenostomatous (narrow buccal cavity and flat dorsal tooth) or eurystomatous (wide buccal cavity and barbed dorsal and ventral teeth) according to Bento et al 2010 [10]. A total of at least 45 animals from 5 dauer populations in each category were observed for mouth form and animals whose mouth forms cannot be classified were not included in the ratio count.

**Nile Red Fat Staining**

We utilized the previously described method of using the lipophilic dye Nile Red (Sigma-Aldrich, N3013) to detect the presence of triacylglycerides and phospholipids in the intestines of fixed animals [27]. Detection of fat storage by Nile Red in live worms is unreliable due to lysosome processing but detection in fixed specimen have been shown to be reproducible and correlated with fat detection by TLC/GC [39,27]. In brief, nematode cultures were washed twice with M9 buffer and freeze-thawed thrice (liquid nitrogen/30°C water bath) in 500 μl of 0.5% freshly diluted paraformaldehyde in M9 buffer (w/v). The fixed worms were then washed twice with 500 μg/ml of freshly diluted Nile Red solution in PBS buffer and incubated at room temperature for 45–60 minutes. The stained worms were subsequently washed twice with 3x volumes of M9 buffer and placed on 2% Noble agar pads on glass slides for fluorescence microscopy using a Leica DM6000 with the cy5 filter. At least 3 independently grown cultures were analyzed per nematode genotype per food type. Both the size and intensity of stained lipid droplets have been found to correlate with fat levels. However, we found the size of the lipid droplets to be more consistent than the intensity of staining within genotypes. Therefore we measured the diameters

| Behavior                  | egl-4 | obi-1 | egl-4:obi-1 |
|---------------------------|-------|-------|-------------|
| % Forward                 | ↑     | –     | ↓           |
| % Reverse                 | –     | ↓     | ↓           |
| % Stopped                 | –     | –     | ↑           |
| Forward Velocity          | –     | –     | ↓           |
| Reversal Frequency        | –     | –     | ↓           |
| % Stopped                 | –     | –     | ↓           |

*Table 2. Changes in pharyngeal pumping rate in Ppa-egl-4 and Ppa-obi-1 mutants compared to wild-type P. pacificus.*

*Table 3. Changes in locomotion on/off E. coli OP50 in Ppa-egl-4 and Ppa-obi-1 mutants compared to wild-type P. pacificus.*

*Table 3. Changes in locomotion on/off E. coli OP50 in Ppa-egl-4 and Ppa-obi-1 mutants compared to wild-type P. pacificus.*

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*Table 3. Changes in locomotion on/off E. coli OP50 in Ppa-egl-4 and Ppa-obi-1 mutants compared to wild-type P. pacificus.*
of the two largest fluorescent droplets in the medial anterior portion of the intestine (buccal cavity in focus and half way between the pharyngeal-intestinal valve and the invaginating vulva, approximately 50 μm) of L4/J4 stage hermaphrodites cultured on 3 day-old E. coli OP50 or C. crescentus NA1000 lawns. The mean values of these lipid droplets were expressed as volumes (N×10^4 μm^3) using the Leica LAS imaging analysis software.

**Nematode Locomotion and Data Analysis**

Worms were tested by automated tracking continuously cultured on E. coli OP50, and tested on OP50. We used 10 cm non-seeded NGM plates to test different parameters of locomotion. Worms were placed on assay plates containing either a 2 cm lawn of an overnight culture of E. coli OP50 or plates not containing any food [36]. As previously described, 10 cm NGM plates used for recordings were equilibrated to 20°C for 18–20 hours. Approximately one hour before beginning recordings, 600 μl of fresh OP50 overnight culture was spread on each plate to achieve a thin, featureless lawn of food across the entire surface. Excess solution was drawn from the edge with a Pipetman. Food was allowed to dry on the agar surface of a tissue paper-covered plate until the surface exhibited a matte finish (about 45 minutes). L4/J4 hermaphrodites of both C. elegans and different mutations of P. pacificus were picked to freshly seeded plates 16–20 hours prior to recording. Individual worms were transferred to assay plates and the plate placed in a holder on the microscope stage. After two minutes of recovery, the worm was located and recording begun using an automated worm tracker and image recorder specially designed for studying worm locomotion [36,37]. Each worm was recorded for five minutes. Data extraction, processing and analysis were done using image processing and analysis software as previously described [36,37]. From each video recording of 5 minutes, we used the middle 4 minutes, and used the software to derive values for frequency of undulations. All incubations and recordings were done in a constant temperature room at 20°C.

**Statistical Analysis**

Means, SEM (error bars), and P values for two-tailed t-test were performed by Microsoft Excel. Significant P values in figures are denoted by *: P<0.05; **: P<0.01; ***: P<0.001. One-way ANOVA, Tukey-Kramer and Dunnett’s Multiple comparisons post-hoc testing were performed using the InStat statistical software.

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**Author Contributions**

Conceived and designed the experiments: RLH JS. Performed the experiments: SMK RJY JS. Analyzed the data: SMK RLH JY JS. Contributed reagents/materials/analysis tools: RLH PWS. Wrote the paper: RLH JS PWS.

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