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RESEARCH ARTICLE

Predatory feeding behaviour in *Pristionchus* nematodes is dependent on phenotypic plasticity and induced by serotonin

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ABSTRACT

Behavioural innovation and morphological adaptation are intrinsically linked but their relationship is often poorly understood. In nematodes, a huge diversity of feeding morphologies and behaviours can be observed to meet their distinctive dietary and environmental demands. *Pristionchus* and their relatives show varied feeding activities, both consuming bacteria and also predating other nematodes. In addition, *Pristionchus* nematodes display dimorphic mouth structures triggered by an irreversible developmental switch, which generates a narrower mouthed form with a single tooth and a wider mouthed form with an additional tooth. However, little is known about the specific predatory adaptations of these mouth forms or the associated mechanisms and behaviours. Through a mechanistic analysis of predation behaviours, in particular in the model organism *Pristionchus pacificus*, we reveal multifaceted feeding modes characterised by dynamic rhythmic switching and tooth stimulation. This complex feeding mode switch is regulated by the neurotransmitter serotonin in a previously uncharacterised role, a process that appears conserved across several predatory nematode species. Furthermore, we investigated the effects of starvation, prey size and prey preference on *P. pacificus* predatory feeding kinetics, revealing predation to be a fundamental component of the *P. pacificus* feeding repertoire, thus providing an additional rich source of nutrition in addition to bacteria. Finally, we found that mouth form morphology also has a striking impact on predation, suppressing predatory behaviour in the narrow mouthed form. Our results therefore hint at the regulatory networks involved in controlling predatory feeding and underscore *P. pacificus* as a model for understanding the evolution of complex behaviours.

KEY WORDS: Predation behaviour, *Pristionchus pacificus*, Neurotransmitter, Developmental plasticity

INTRODUCTION

Animals have evolved different behaviours and morphological adaptations to meet their needs in exploiting different environments. Morphology and behaviour are intimately coupled as behaviour is restricted by morphological limits while also driving morphological diversity through the evolution of novel functions. Nematodes are a well-studied and abundant animal group in which to analyse the relationship between morphology and behaviour. Nematodes show a staggering array of morphological structures and behaviours including a vast spectrum of mouth adaptations that coincide with a great diversity in feeding behaviours and diets (De Ley et al., 1995; von Lieven, 2003; Baldwin et al., 2004; Ragsdale et al., 2008; Sato et al., 2008). In bacteriovorus species such as *Caenorhabditis elegans*, the buccal cavity is often composed of a simple tube with a more complex apparatus found in the terminal bulb where hardened discs of collagen form a grinder to aid in bacterial lysis (Straud et al., 2013). In contrast, parasitic species employ a host of specialised implements for penetrating their host tissue. This includes fine stylets observed in the commercially significant cyst nematodes of the genus *Heterodera* (Handoo, 2002), and sharpened teeth in the medically significant hookworm species *Ancylostoma duodenale* and *Necator americanus* (Hotez et al., 2004, 2010). Omnivorous nematodes including *Pristionchus* are capable of both microbial feeding and predatory feeding on other nematodes, and also have teeth for lacerating open the cuticle of their prey. Interestingly, this mouth specialisation is often dimorphic with one morph, the stenostomatous (St) form thought to be optimised for bacterial food sources and a second morph, the eurystomatous (Eu) form optimised for predation (Serobyan et al., 2013, 2014). *Pristionchus* therefore offers an ideal opportunity to study behaviour under both morphological and behavioural contexts.

The diplogastrid *Pristionchus pacificus* Sommer, Carta, Kim and Sternberg 1996 has been developed as a model organism for comparative and integrative evolutionary biology. It boasts an annotated genome (Dieterich et al., 2008), and phylogenetic and molecular tools (Schlager et al., 2009; Sommer and McGaughran, 2013), and much of its ecology is established, revealing a close necromenic association with scarab beetles (Fig. 1B) (Herrmann et al., 2007). Furthermore, recent mapping of the pharyngeal connectome has exposed fundamental wiring differences between *P. pacificus* and *C. elegans*, perhaps guiding behavioural changes between the species (Bumbarger et al., 2013). The mouth dimorphism in *P. pacificus* has also been intensely studied as a model for developmental (phenotypic) plasticity, thought to be a major process driving novelty in evolution (Susoy et al., 2015). The developmental pathways guiding the mouth dimorphism represent a complex regulatory network including small molecules (Bose et al., 2012). An endocrine signalling pathway consisting of the nuclear hormone receptor *Ppa-daf-12* and the steroid hormone dafachronic acid (DA) act further downstream (Bento et al., 2010). Recently, the sulphatase *eud-1* was identified as a developmental switch that not only has a key function during development but also guides microevolutionary and macroevolutionary patterns (Ragsdale et al., 2013). In contrast to these recently acquired insights into the molecular network regulating the mouth dimorphism in *P. pacificus* (Fig. 1J), very little of the behaviours associated with predatory feeding or the contribution from the differing mouth forms has been studied.

Here, we report a mechanistic analysis of predatory feeding in *P. pacificus*, revealing unique qualities not observable during bacterial feeding. This includes altered pharyngeal dynamics and stimulation of the dorsal tooth, which is used to eviscerate

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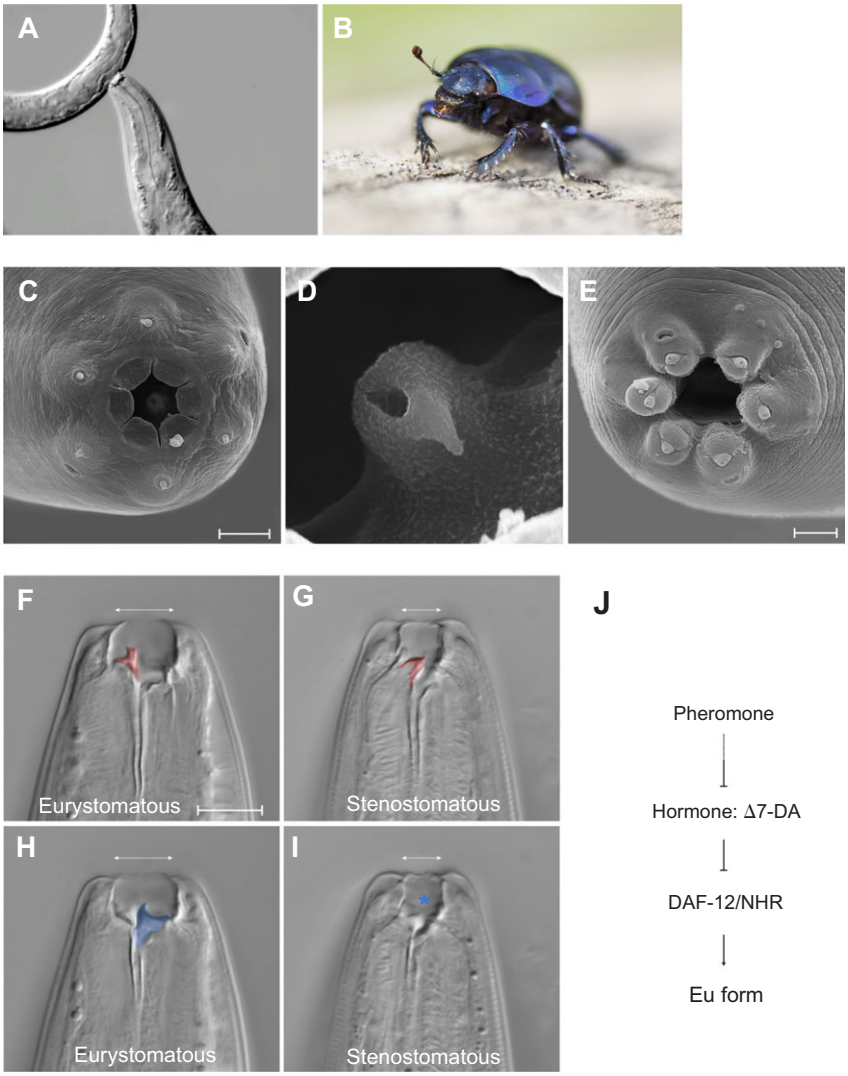

Fig. 1. Developmental plasticity in *Pristionchus pacificus* enables a predatory diet. (A) Image of killing behaviour in a *P. pacificus* eurystomatous (Eu) hermaphrodite feeding on a *Caenorhabditis elegans* larva. Image by Dr Dan Bumbarger. (B) *Pristionchus pacificus* lives in a necromenic association with scarab beetles. Photograph by Jan M. Meyer. (C) SEM images of an adult *P. pacificus* hermaphrodite with Eu mouth form revealing predatory adaptations. Scale bar, 3 µm. (D) Magnified images of the hook-shaped dorsal tooth and the large gland duct opening. (E) SEM image of an adult *C. elegans* hermaphrodite. Scale bar, 2 µm. (F,G) Nomarski image of the mouth dimorphism in *P. pacificus*. A single adult Eu and stenostomatous (St) hermaphrodite across two focal planes. The dorsal tooth, false coloured red, is larger and claw-like in the Eu form (F) compared with the flint-shaped St form (G). (H,I) The opposing subventral tooth, false coloured blue, is large and hook shaped in the Eu form (H), while absent in the St form (asterisk, I). Scale bar in F (applies to F–I), 10 µm. (J) Regulatory model for the mouth form decision pathway. Δ7-DA, Δ7-dafachronic acid; DAF-12 and NHR, nuclear hormone receptors.

prey. Through a pharmacological analysis of the effects of neurotransmitters, we identified a functional basis for regulating the behavioural switch between bacterial feeding and predatory feeding, and show this is conserved across several predatory nematode species. Furthermore, to understand predator and prey interactions, we investigated the impact of starvation, prey size and prey preference on *P. pacificus* predatory feeding. Finally, as the *Pristionchus* genus is well characterised, with most species revealing phenotypically plastic mouth structures falling into two distinct mouth types, we exploited this phenomenon to investigate the effects of mouth form morphology on predatory success and on predatory feeding behaviour itself.

RESULTS

Predatory feeding mode induces altered feeding rhythms

Pristionchus pacificus, like many diplogastrids, is capable of predatory feeding behaviour, supplementing its available food by feeding on other nematodes (Fig. 1A; supplementary material Movie 1). Accompanying this behaviour, a mouth dimorphism exists in *P. pacificus*, with the St bacteriovorus mouth comprising a single blunt dorsal tooth and the Eu predatory mouth form composed of a pair of opposing teeth including a flexible and enlarged dorsal tooth and a sizeable subventral tooth (Fig. 1C–I). Mouth forms can be readily identified based on these morphological differences. Furthermore, the pharynx of *P. pacificus* can be divided into three regions as in *C. elegans*, based on pharyngeal muscle groupings. These are the corpus, isthmus and terminal bulb. While in *C. elegans* pumping occurs anteriorly in the corpus and anterior isthmus and posteriorly in the terminal bulb, in *P. pacificus* pumping occurs only anteriorly in the corpus (Chiang et al., 2006). To explore predatory feeding behaviour in *P. pacificus*, we first analysed pharyngeal rhythms and the effect on tooth stimulation under different feeding conditions. We found that pumping rates during bacterial feeding were substantially higher than during predatory feeding (Fig. 2A). In contrast, the movement of the dorsal tooth could be observed only sporadically during bacterial feeding. However, tooth movement increased substantially during predatory feeding and appeared to be coupled to pharyngeal pumping in a 1:1 ratio (Fig. 2A). Thus, *P. pacificus* shows considerable differences in its feeding rhythms and dynamics during bacterial and predatory feeding.

Predatory feeding rhythms can be stimulated through serotonin exposure

To elucidate the neurobiological basis underlying the regulation of feeding behaviour, we conducted pharmacological experiments analysing the effects of the neurotransmitters serotonin, dopamine, octopamine and tyramine on pharyngeal pumping and tooth

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movement rate (Fig. 2B). Of these neurotransmitters, only serotonin triggered a predatory-like pumping and tooth movement response, causing a reduction in pharyngeal rhythms and stimulating tooth activity to levels similar to those observed while feeding on prey (supplementary material Movie 2). Despite lacking the opposing teeth of the Eu mouth form, St morph animals still possess a noticeable dorsal tooth although with very different morphology. Because of this evident difference in tooth morphology, we questioned whether it was controlled by a similar process. Wild-type *P. pacificus* (PS312) is mostly of the Eu morph with low numbers of St morph animals detectable. Fortunately, the recently characterised *eud-1* mutant (Ragsdale et al., 2013) is entirely St and therefore provided a means to acquire abundant St mouth form animals. Exposure of St animals to serotonin also elicited a strong predatory-like response, with tooth movement and pumping rates replicating those observed in Eu morph animals during predatory feeding (Fig. 2A). Thus, serotonin induces similar predatory-like feeding behaviour in both Eu and St morph animals.

Serotonin control of predatory feeding rhythms is evolutionarily conserved

As serotonin had such a profound effect on the feeding dynamics of *P. pacificus*, inducing predatory feeding rhythms, we speculated that this effect may be conserved among other predatory nematodes. In addition to *P. pacificus*, predation is a common feeding mechanism utilised by many other nematode species including other members of the genus *Pristionchus* but also many of its more distant relatives. Therefore, we tested three further *Pristionchus* species, including the closely related sister species *P. exspectatus*, as well as *P. entomophagus* and *P. fissidentatus* and a more distantly related representative outgroup *Allodiplogaster sudhausi*. In all assayed species, serotonin induced tooth stimulation in a 1:1 ratio with pharyngeal pumping, indicating a conserved mode of action (Fig. 2C). It is therefore conceivable that this mechanism of serotonin mediated predatory feeding mode switching may be conserved amongst diverse predatory nematodes.

Serotonin in *C. elegans* has long been associated with food signalling, increasing pharyngeal pumping rate while slowing locomotion. Consequently, the associated serotonergic neurons have been well characterised through numerous antibody and expression studies (Horvitz et al., 1982; Avery and Horvitz, 1990; Sze et al., 2000). In order to elucidate potential neurons associated with the modulation of predatory rhythms, we conducted serotonin immunostaining in *P. pacificus*. Antibody staining indicated several serotonergic neurons situated in the pharynx of *P. pacificus* as previously reported (Rivard et al., 2010). One pair of neurons appeared strongly serotonergic (Fig. 2D). These neurons were identified as the neurosecretory motor neurons (NSM), which are known to be strongly serotonergic in *C. elegans*. A second more anterior pair of neurons displayed weaker serotonin expression and, based on their position and form, they were identified as the pharyngeal interneurons, I1. Interestingly, the I1 neurons in *P. pacificus* were identified as a candidate regulator of predatory feeding in a recent connectomic study (Bumbarger et al., 2013).

Kinetics of predator–prey interactions

To study the interactions between *P. pacificus* predators and *C. elegans* prey, we initially focused on the Eu mouth morph because of its previous predatory associations (Serobyan et al., 2014). Predatory feeding behaviour was divided into three distinct categories. Firstly, 'bites', defined by a switch into predatory pumping rhythms and coinciding with a restriction in motility of the prey species. This was often accompanied by a strong touch response in the prey when the bite was unsuccessful. Secondly, 'kills', as indicated by an opening of the prey cuticle and leaking of pseudocoelomic fluid. Thirdly, 'feeding' events, categorised by the predator successfully opening the prey cuticle and remaining in close proximity to the laceration, consuming leaking innards. This was frequently accompanied by switching between bacterial and predatory pumping rhythms. Upon transferral and recovery of *P. pacificus* predators to biting assay plates (supplementary material Fig. S1), biting behaviour rapidly commenced, with an average of 15.3±6.2 bites per 10 min. Of these bites, 5.3±3.1 resulted in successful kills during the 10 min assay, an average killing efficiency of 35%. Surprisingly, not all bites were accompanied by feeding behaviour, with an average 2.6±2.2 feeding events per 10 min, corresponding to only 49% of successfully killed prey (Fig. 3A). This may be caused in part by prey being damaged as they escape or intriguingly may be evidence that *P. pacificus* predators eliminate potential competition.

To clarify whether the predatory feeding behaviour observed in *P. pacificus* is induced more or less strongly upon starvation, *P. pacificus* predators were starved for 4, 8, 24, 48 and 72 h before being placed with *C. elegans* larvae and repeating the previous assay (Fig. 3A). For each of the three feeding behaviour types quantified (i.e. biting, killing and feeding), a weak to moderate negative correlation was observed between the number of times the behaviour was expressed and starvation time (Pearson product-moment correlation, $r_{58}=-0.58$, $P<0.001$ for biting; $r_{58}=-0.39$, $P=0.002$ for killing; $r_{58}=-0.27$, $P=0.04$ for feeding). After a 4 h starvation, a minor increase in biting behaviour was elicited, possibly representing some predatory modulation of biting behaviour during early starvation. This was, however, absent at the later starvation time points. More noticeable was the decrease in biting events after severe starvation times such as after 72 h without food. Although these animals showed a reduction in biting activity, behaviourally animals spent prolonged periods feeding on killed individual prey, ensuring maximum uptake of nutrients and therefore reducing the total number of bites. Taken together, starvation is therefore not necessary to induce predatory feeding behaviour and, consequently, is likely to form part of the *P. pacificus* conventional feeding repertoire, probably supplementing its available bacterial nutrient intake with nematodes whenever possible.

Pristionchus has a preference for bacterial food

Next, we investigated aspects of *P. pacificus* predatory feeding resembling more closely the conditions found in nature, such as co-existence of food sources and differently staged prey items. It is highly likely that both bacteria and nematode larvae are represented in the regular diet of *P. pacificus* worms and it is also probable that *P. pacificus* may encounter the two food sources concurrently. Under such circumstances, food preference and choice may influence *P. pacificus* feeding behaviour. We therefore analysed which food source *P. pacificus* preferred through a chemoattraction assay between nematode larvae and a bacterial food source. As our laboratory *P. pacificus* strains are successfully raised on *Escherichia coli* OP50, we selected this as our bacterial food source and utilised immobilised *C. elegans* larvae as a counter-option. Under assay conditions, *P. pacificus* predators appeared to favour the bacterial food source with a chemoattraction index of 0.55. As *P. pacificus* appeared to have a preference for bacteria over nematode larvae, we next tested the impact of this on predatory feeding behaviour. *Pristionchus pacificus* predators were placed on an assay plate containing both a lawn of *E. coli* and *C. elegans* larvae and the

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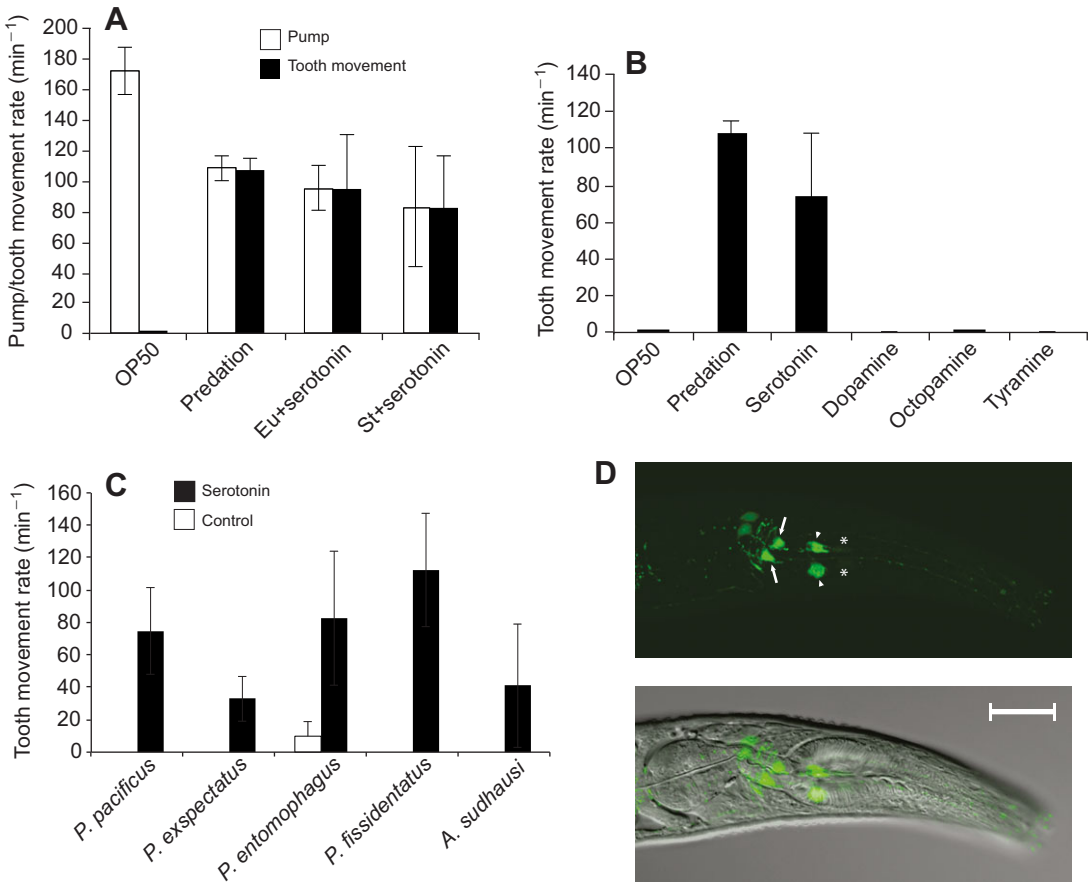

Fig. 2. Predatory feeding mode is regulated by the neurotransmitter serotonin. (A) Quantification of pharyngeal pumping and tooth movement rates reveals significant differences between bacterial and predatory feeding. Pumping rate is substantially lower during predatory feeding compared with bacterial feeding, while tooth movement rate increases dramatically. The neurotransmitter serotonin (10 mmol l^{-1}) triggers a predatory-like pumping and tooth movement response in both Eu (wild-type) and St (*eud-1* mutant) animals. OP50, *Escherichia coli* OP50. Error bars represent s.d. (B) Neurotransmitter effect on tooth movement rate. Treatment with 10 mmol l^{-1} serotonin triggers a predatory-like tooth movement response. The neurotransmitters dopamine (10 mmol l^{-1}), octopamine (10 mmol l^{-1}) and tyramine (10 mmol l^{-1}) do not affect tooth movement. Error bars represent s.d. (C) Serotonin (10 mmol l^{-1}) effect on tooth movement rate in Eu animals of selected *Pristionchus* species and the outgroup *Allodiplogaster sudhausi*. Serotonin triggers a predatory-like pumping and tooth movement response in all predatory nematodes tested. Error bars represent s.d. (D) Serotonin immunostaining merged with DIC image in whole-mount heads of adult nematodes. Anterior is to the right. Serotonergic neurons, probably corresponding to NSM (arrowheads), ADF (arrows) and I1 (asterisks), could be detected in the pharynx of *P. pacificus*. Scale bar, 20 µm.

number of biting events during a 10 min interval was observed. The number of biting events while *P. pacificus* predators were surrounded by bacteria was significantly lower (t_{18}=−4.183 P=0.0006) than when they were without bacteria, therefore reinforcing the preference for this bacterial food source over *C. elegans* nematode larvae (Fig. 3B). However, although the biting of larvae was lower, biting and killing events were still abundant. In its natural environment, *P. pacificus* is likely to encounter nematodes of all developmental stages. To understand the predatory capabilities of *P. pacificus*, we challenged *P. pacificus* predators with *C. elegans* prey of different developmental stages. *Pristionchus pacificus* predators were fed on *C. elegans* larvae immediately post-filtering, as well as after 24, 48 and 72 h of growth on OP50 (Fig. 3C). *Caenorhabditis elegans* larvae grown for 24 and 48 h were still in larval stages, whereas 72 h of growth results in gravid adults. Biting behaviour and successful kills were observed across all time points. *Pristionchus pacificus* predators were able to successfully bite all developmental stages of *C. elegans* with a consistent biting rate; no association between the number of bites and prey size was observed (Pearson product-moment correlation, r_{38}=−0.11, P=0.49). Killing efficiency, however, was negatively correlated with the prey size (r_{38}=−0.64, P<0.001) when the 72 h post-filter time point was included, and showed no correlation when only the first three time points were included in the analysis (r_{38}=−0.03, P<0.88). We hypothesise that at 72 h post-filtering, the *C. elegans* cuticle had probably reached a suitable thickness to prevent *P. pacificus* penetrating and successfully killing them. Despite this, *P. pacificus* bites still elicited a strong touch response in the 72 h *C. elegans* prey, inducing noticeable discomfort (supplementary material Movie 3).

Eu mouth form is required for predatory feeding behaviour in the genus *Pristionchus*

Nearly all members of the genus *Pristionchus* are dimorphic (*P. elegans* is fixed in the St morph while *P. triformis* has an additional extreme mouth morph, megastomatous, Mg), and so presented an ideal opportunity to study the influence of mouth morphology on predatory feeding behaviour within an evolutionary context. We selected 20 *Pristionchus* species and four additional diplogastrid species as comparative out-groups with which to analyse the influence of mouth form on predatory feeding behaviour (Fig. 4A). Animals were selected for mouth form, and corpse assays

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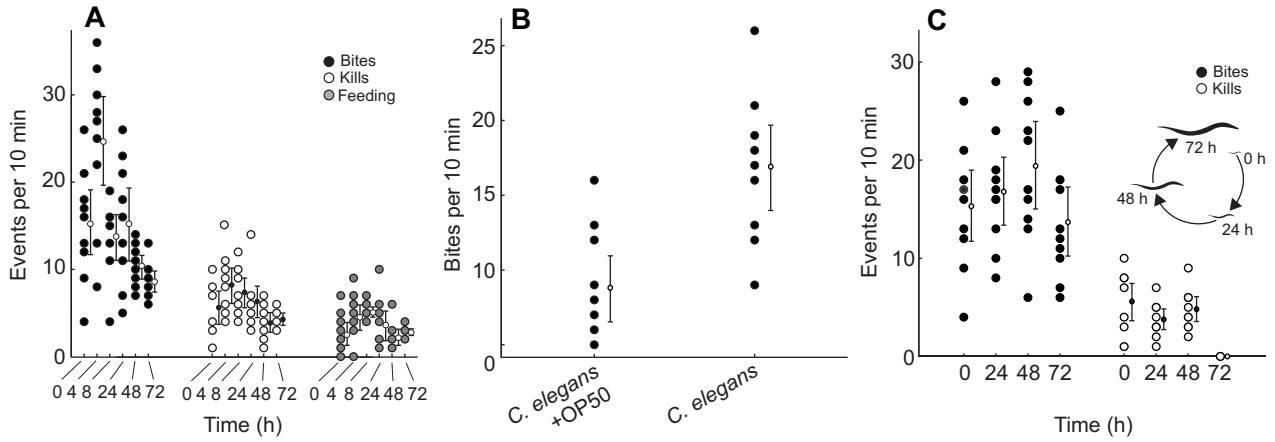

Fig. 3. Predator–prey interactions. (A) Biting assay investigating the effects of starvation upon *P. pacificus* predatory behaviour. *Pristionchus pacificus* predators were starved for 4, 8, 24, 48 h and subsequently fed on *C. elegans* prey. Bites, kills and feeding events were quantified (*N*=10). Starvation initially modulates an increase in biting activity, with severe starvation inducing prolonged feeding times that reduce the number of bites. Error bars represent 95% confidence interval of the mean. (B) Biting assay examining *P. pacificus* predators feeding on *C. elegans* larvae alone and also on *C. elegans* larvae in the presence of *E. coli* OP50. In the presence of additional bacterial food, *P. pacificus* biting rates decreased although biting of larvae was still abundant. (C) Biting assay examining the effects of prey size on predation success. *Pristionchus pacificus* were transferred to plates containing filtered *C. elegans* larvae fed for 0, 24, 48 and 72 h encompassing L1 to adult prey, and biting behaviour together with successful kills were quantified. *Pristionchus pacificus* bites all developmental stages of *C. elegans* prey with only adult animals protected (*N*=10). Each point represents a single replicate. If fewer than 10 circles are visible, they are hidden behind those displayed.

(supplementary material Fig. S1) were carried out on each species and mouth morph to detect successful predation events. Strikingly, corpses were not observed on any *Pristionchus* St assay plates while corpses were detected on nearly all Eu assay plates (Fig. 4B). *Pristionchus* St mouth forms were therefore unable to undertake predatory feeding, indicating a strong morphological influence on behaviour. In contrast, in all out-group species tested, St animals were still able to kill at a similar frequency to the other mouth morphs. It is important to note that all tested out-groups have St mouth forms that have a dorsal and subventral tooth, whereas the latter is absent in St animals of *Pristionchus* species.

Although *Pristionchus* St mouth forms were unable to kill prey, it was unclear whether this was caused by a mechanical deficiency, perhaps due to the much narrower mouth cavity in St animals, or if the killing behaviour itself was altered. Consequently, we selected four *Pristionchus* proficient killers and the out-group species *A. sudhausi* for further analysis via bite assay (Fig. 4C). As expected, both Eu and St mouth morphs of *A. sudhausi* frequently attacked prey. However, in all *Pristionchus* species tested, only the Eu mouth morph animals engaged in predatory feeding behaviour (supplementary material Movie 1). St mouth morphs appeared uninterested in the surrounding prey and no bites were detected (supplementary material Movie 4).

St mouth morphs are opportunistic scavengers

As we had previously demonstrated feeding mode switching in both *P. pacificus* Eu and St animals through exposure to serotonin (Fig. 2A), the finding that St morph animals failed to kill prey was somewhat surprising. In nature, Eu and St animals are likely to co-exist. As Eu mouth morphs kill abundantly, we speculated that St mouth morphs might be able to utilise a predatory feeding mode for scavenging on the corpses of killed nematodes. Consequently, we placed several adult *C. elegans* corpses amongst *P. pacificus* St animals. Indeed, predatory feeding behaviour was immediately stimulated upon contact with the opened *C. elegans* corpses (Fig. 4D). These findings indicate that predatory feeding behaviour is robustly suppressed in *Pristionchus* St animals; however, it is not entirely abolished, providing St animals with a mechanism for the opportunistic scavenging of nematode corpses.

DISCUSSION

This study is the first to characterise predatory feeding behaviour in nematodes, exposing fundamental differences between bacterial and predatory feeding dynamics and, most significantly, revealing a functional role for serotonin in this process as well as demonstrating the influence of mouth morphology on behaviour. We show that *P. pacificus* feeding dynamics are heavily influenced by diet, with distinct pumping rates and tooth stimulation corresponding directly with bacterial or predatory feeding. *Pristionchus pacificus* bacterial feeding is associated with rapid pharyngeal pumping (Kroetz et al., 2012) while we show predatory feeding necessitates slower, stronger pharyngeal rhythms coinciding with tooth activity in a 1:1 ratio. These differing feeding rhythms fall into discrete categories probably with differing genetic, muscular and neuronal contributions. As *P. pacificus* and *C. elegans* share identical numbers of pharyngeal neurons and mapped pharyngeal positions (Bumbarger et al., 2013), future cell-ablation experiments will help dissect the neuronal networks involved.

Feeding mode switching to predatory feeding behaviour appears to be driven by the neurotransmitter serotonin, although the genetic and neurobiological mechanisms behind this are as yet unknown. The effect of serotonin on nematode behaviour has been extensively studied in *C. elegans* by coupling food sensation to various neurological processes while identifying the neurons associated (Horvitz et al., 1982; Avery and Horvitz, 1990; Sze et al., 2000). Within the pharynx, only the NSM neurons are highly serotonergic in *C. elegans* and have a postulated role in food signalling. In the pharynx of *P. pacificus*, a pair of strongly serotonergic neurons was identified similar to the findings of a previous report (Rivard et al., 2010), corresponding to the NSM neurons, with a pair of weak serotonergic neurons anterior to this. These additional serotonergic neurons correspond to *P. pacificus* I1, which reinforces prior

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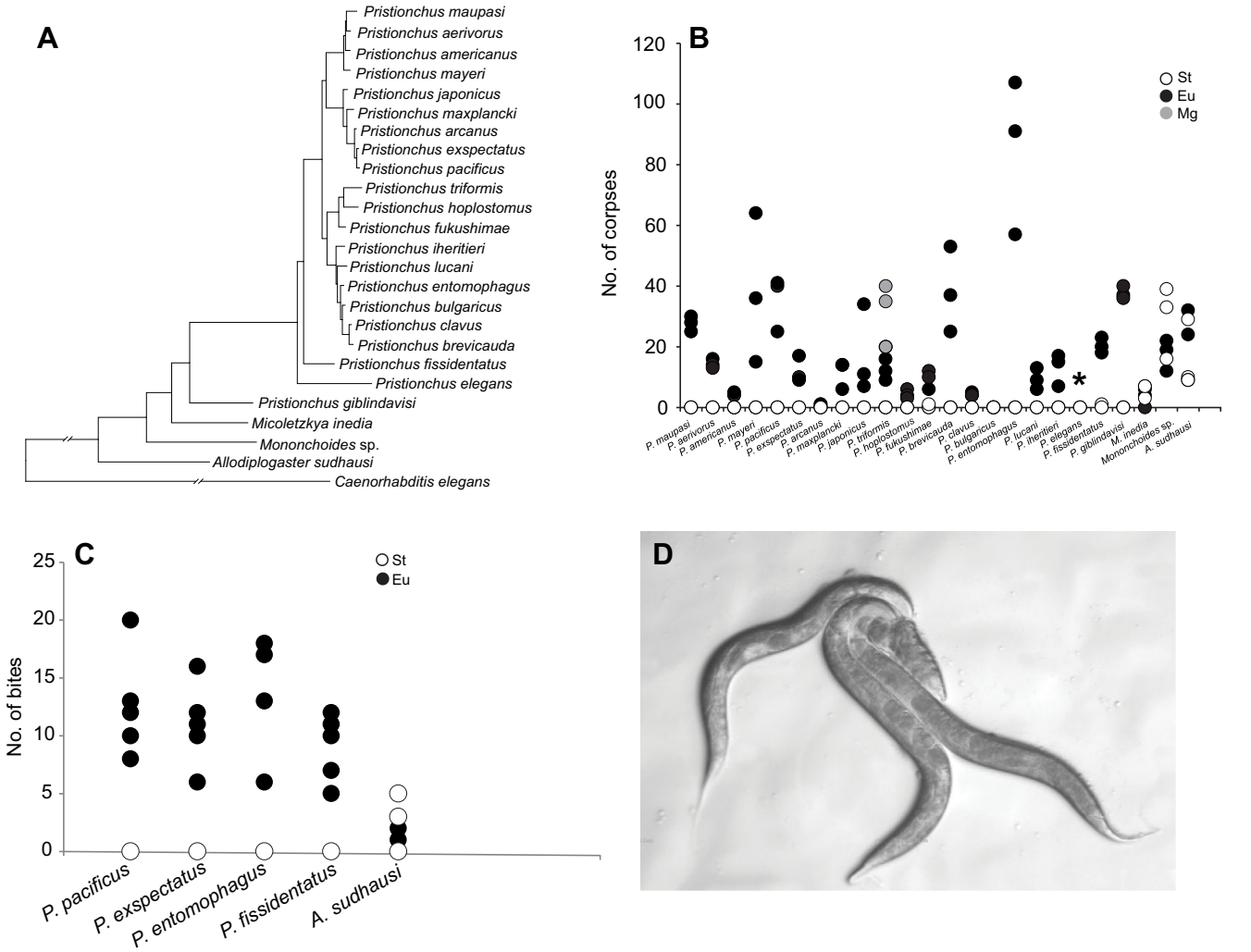

Fig. 4. Eu mouth form is required for predatory feeding behaviour in the genus *Pristionchus*. (A) Maximum likelihood tree of *Pristionchus* and outgroup species examined for predatory feeding behaviour on filtered *C. elegans* larvae, inferred using partial 18 S rRNA and 27 ribosomal protein genes. (B) Corpse assays investigating the effects of mouth form on predatory feeding success. Eu and St mouth forms for each species were analysed independently in triplicate for *N*=5 animals and the corpses counted after 2 h. Predatory feeding behaviour is prevalent in the majority of species analysed. No corpses were visible on St plates of any species. *The monomorphic *P. elegans* with a fixed St mouth form (*N*=3). Mg, megastomatous. (C) Bite assay of selected efficient predatory killing species in order to differentiate between a failure to successfully kill prey and a failure to engage in predatory feeding behaviour. Predatory behaviour appears suppressed in St mouth forms of *Pristionchus* species (*N*=5). Each point represents a single replicate. If fewer circles are visible than specified, they are hidden behind those displayed. (D) St mouth forms engage in predatory-like feeding rhythms, facilitating the scavenging of nematode corpses.

connectomic data speculating additional predatory functionality of this neuron pair based on graph theory analysis (Bumbarger et al., 2013). Furthermore, with recent advancements in methods for generating gene knockouts through CRISPR generated mutations (Lo et al., 2013; Witte et al., 2015), the analysis of genes involved in components of neurotransmitter synthesis and receptor genes can now be investigated.

While studies on the dimorphic properties of the *P. pacificus* mouth form have made great strides in elucidating the intricate developmental and chemical queues driving this process (Bento et al., 2010; Bose et al., 2012; Ragsdale et al., 2013), little was known of the influence of mouth form morphology on behaviour or its evolutionary conservation. Our study reveals a strong correlation between mouth morph and predatory feeding behaviour conserved within the *Pristionchus* genus. Animals with the smaller St mouth morph are unable to bite or kill live prey, indicating robust suppression of predatory killing behaviour. Despite the lack of killing behaviour in St morph animals, the predatory feeding mode was retained and still utilised for the opportunistic scavenging of previously killed nematodes. Our studies also show the importance of differentiating between predatory feeding behaviour and scavenging behaviour, perhaps explaining discrepancies with a previous finding (Serobyan et al., 2014). In this context, we would speculate that the observed killing without feeding behaviour of Eu and the scavenging behaviour of St morphs might be of significance under real environmental conditions. Additionally, the killing of nematodes by Eu morph animals leaves abundant carcasses, which are likely to stimulate the growth of more bacteria, thus providing additional nutritional benefits for the nematodes, while the elimination of competitors might represent a second function for the killing behaviour of Eu animals; support for such hypotheses will await testing in real ecological settings.

Taken together, our results characterised predatory feeding behaviour in *P. pacificus*, revealing novel functions for the well-described neurotransmitter serotonin. In addition, through our analysis of the mouth form dimorphism present in most

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Pristionchus species we also implicated this developmental decision in the control of predatory behaviour, indicating the existence of additional regulatory pathways feeding into this neuronal system. Therefore, further investigations into *P. pacificus* predatory feeding behaviour should provide a greater understanding of the mechanisms driving the evolution of complex behaviours within fixed neuronal networks.

MATERIALS AND METHODS

Nematode species

Nematode species were maintained at 20°C on NGM agar plates with *E. coli* OP50. Worm species and strains used were *C. elegans* (N2), *P. pacificus* (PS312), *P. maupasi* (RS0143), *P. aerivorus* (RS5106), *P. americanus* (RS0140), *P. mayeri* (RS5460), *P. exspectatus* (RS5522B), *P. arcanus* (RS5527), *P. maxplancki* (RS5594), *P. japonicus* (RS5528), *P. triformis* (ST5233), *P. hoplostomus* (JU1090), *P. fukishimae* (RS5595), *P. brevicauda* (RS5231), *P. clavus* (RS5284), *P. bulgaricus* (RS5283), *P. entomophagus* (RS0144), *P. lucani* (RS5050), *P. inheritieri* (SB245), *P. elegans* (RS5229), *P. fissidentatus* (RS5133), *P. giblindavisi* (RS5555), *Micoletzkya inedia* (RS5605), *Mononchoides* sp. 1 (RS5441) and *A. sudhausi* (B413).

Pumping and tooth rate

Pristionchus pacificus predators were maintained at 20°C until young adult stage. Animals were then transferred to an assay plate seeded with OP50 or filled with >5000 *C. elegans* larvae for observation via a Zeiss Axio Imager a1 microscope and high speed camera. Animals were left undisturbed for 15 min prior to assaying to allow for recovery. Pharyngeal pumping and tooth movements were observed for 15 s in at least 20 animals under each condition. *Caenorhabditis elegans* prey were prepared by growing cultures until newly starved and therefore contained an abundance of L1 and L2 larvae. These plates were washed with M9 and then passed through two 20 µm filters to remove larger animals. The filtered wash was centrifuged at 1500 rpm and 2 µl of the larval pellet added to each 6 cm NGM unseeded assay plate before the addition of any *P. pacificus* predators. For pharmacological assaying of neurotransmitters, animals were transferred to small (3 cm) assay plates containing 10 mmol l^{-1} of the appropriate neurotransmitter, and pharyngeal pumping and tooth movement were observed as described above.

Immunohistochemistry

Staining with anti-serotonin antibody was carried out as described by Desai et al. (1988). Briefly, worms were rinsed 3× in M9 and fixed overnight at 4°C in 500 µl of 4% paraformaldehyde/PBS. After being washed 3× in 0.5% Triton X-100/PBS, the animals were incubated overnight at 37°C in 500 µl of 5% β-mercaptoethanol/1% Triton X-100/0.1 mol l^{-1} Tris, pH 7.4. After incubation, worms were further washed 2× in 1% Triton X-100/0.1 mol l^{-1} Tris, pH 7.4, followed by a wash in 1× in collagenase buffer (1 mmol l^{-1} $CaCl_2$/1% Triton X-100/0.1 mol l^{-1} Tris, pH 7.4). They were then incubated for 2–8 h at 37°C in collagenase type IV in collagenase buffer. Animals were centrifuged at 1500 rpm and the pellet washed 3× in 0.5% Triton X-100/PBS before blocking for 1 h at room temperature in 100 µl of 1% BSA/0.5% Triton X-100/PBS. Processed animals were incubated overnight at room temperature in 1:100 anti-serotonin antibody in 1% BSA/0.5% Triton X-100/PBS before washing a further 2× in Triton X-100/PBS to remove unbound antibodies. A second blocking reaction for 1 h in 0.1% BSA/0.5% Triton X-100/PBS was carried out before incubation overnight at 4°C in 50 µl of 1:100 cy3 goat anti-rabbit secondary antibody. Finally, excess secondary antibody was removed by washing 3× in 0.5% Triton X-100/PBS and the worms mounted with Vectashield for imaging. Images were acquired on an Olympus FV 1000 confocal microscope.

Mouth form phenotyping

Mouth form phenotyping was attained through observation of the buccal cavity via a Zeiss SteREO Discovery V12 microscope. Morph identities were categorised based on previous described species characteristics and could be subsequently verified through mounting and ×40 Nomarski examination.

Biting assay

Biting assays provided more detailed behavioural analysis. Prey were maintained on bacteria until freshly starved, resulting in an abundance of young larvae. These plates were washed with M9, passed twice through a 20 µm filter, centrifuged and deposited on to the assay plate by pipetting 2 µl of worm pellet on to a 6 cm NGM unseeded plate. Predatory nematodes were screened for the required mouth morph and a single classified predator placed on to the assay plate and allowed to recover for 15 min. After recovery, the predatory animal was observed on a light stereo-microscope for 10 min and the number of bites/kills/feeding events quantified as appropriate. Bites were characterised by a switch in predator pumping mode coincident with a restriction in movement of the prey; kills were characterised by an opening of the prey cuticle; and feeding was characterised by the predator remaining adjacent to the successfully lacerated prey and observable consumption taking place (supplementary material Fig. S1A). Each assay was conducted 5–10 times depending on the experiment to ensure repeatability. The associations between different types of feeding behaviour and independent variables (i.e. starvation time and prey size) were tested using Pearson product-moment correlation. Size data for *C. elegans* were retrieved from Fernando et al. (2011).

Corpse assay

Corpse assays facilitated rapid quantification of predatory behaviour over a large number of species. Prey were maintained on bacteria until freshly starved, resulting in an abundance of young larvae. These plates were washed with M9, passed twice through a 20 µm filter, centrifuged and deposited on to the assay plate by pipetting 2 µl of worm pellet on to a 6 cm NGM unseeded plate. Five predatory nematodes were screened for the appropriate mouth morph and added to assay plates. Predators were permitted to feed on the prey for 2 h and the plate was subsequently screened for the presence of deflated corpses (supplementary material Fig. S1B). Each assay was conducted in triplicate to ensure repeatability.

Chemoattraction assay

Chemoattraction assays were carried out similar to previously described *Pristionchus* procedures with some modifications (Hong and Sommer, 2006). Briefly, standard nematode growth plates were seeded with a small spot of OP50 or with filtered *C. elegans* larvae at either side of the plate. To each attractant spot, 1.5 µl of 1 mol l^{-1} sodium azide was added in order to anaesthetise worms and prevent them from leaving the area upon attraction. Twenty *P. pacificus* adult worms were added equidistant between the two attractant sources and left for 16 h. The number of animals located at each source was quantified and a chemotaxis index scored by the following calculation: (no. of animals at attractant−no. of animals at control) / (total no. of nematodes at attractant and control). Three replicates were carried out and the mean chemoattraction index of the replicates was calculated.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

All experiments performed by M.W., J.W.L. and V.S. M.W., J.W.L. and R.J.S. designed the experiments and wrote the manuscript.

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Supplementary material

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