

Video Article

Assaying Predatory Feeding Behaviors in *Pristionchus* and Other Nematodes

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Abstract

This protocol provides multiple methods for the analysis and quantification of predatory feeding behaviors in nematodes. Many nematode species including *Pristionchus pacificus* display complex behaviors, the most striking of which is the predation of other nematode larvae. However, as these behaviors are absent in the model organism *Caenorhabditis elegans*, they have thus far only recently been described in detail along with the development of reliable behavioral assays¹. These predatory behaviors are dependent upon phenotypically plastic but fixed mouth morphs making the correct identification and categorization of these animals essential. In *P. pacificus* there are two mouth types, the stenostomatous and eurystomatous morphs², with only the wide mouthed eurystomatous containing an extra tooth and being capable of killing other nematode larvae. Through the isolation of an abundance of size matched prey larvae and subsequent exposure to predatory nematodes, assays including both "corpse assays" and "bite assays" on correctly identified mouth morph nematodes are possible. These assays provide a means to rapidly quantify predation success rates and provide a detailed behavioral analysis of individual nematodes engaged in predatory feeding activities. In addition, with the use of a high-speed camera, visualization of changes in pharyngeal activity including tooth and pumping dynamics are also possible.

Video Link

The video component of this article can be found at <http://www.jove.com/video/54404/>

Introduction

Nematodes with their small but complex nervous systems have proved powerful tools for understanding many aspects of neurobiology including behavior. Much of this research has focused on the model organism *Caenorhabditis elegans* in which a wealth of different behaviors have been successfully dissected and analyzed. These include mechanosensory³, chemotactic⁴, thermotactic^{5,6} and magnetotactic⁷ influencing mating^{8,9}, learning¹⁰ and feeding behaviors¹¹. However, other more distantly related nematode species display behaviors which are not observed in the rhabditid *C. elegans* or alternatively show additional levels of complexity, which raises pertinent questions regarding their evolution and regulation. One such instance of this can be observed in the distantly related diplogastrid nematode *Pristionchus pacificus*, which displays much more complex feeding behaviors and rhythms than are observed in *C. elegans*¹. This is despite the two species sharing homologous pharyngeal neurons¹². Coinciding with these additional feeding behaviors, *P. pacificus* also displays an expanded dietary range, as they are avid predators, capable of supplementing their bacterial diet by also feeding on the larvae of other nematodes. Fortunately, *P. pacificus* has been developed as a model for comparative and integrative evolutionary biology and therefore many molecular and genetic tools are now available. These include a fully sequenced and annotated genome¹³, molecular and genetic tools including transgenes¹⁴ and CRISPR/Cas9^{15,16} as well as a detailed and well annotated phylogeny¹⁷ with over 25 closely related species including its newly discovered sister species. In addition, the ecology of numerous *Pristionchus* species including *P. pacificus* is well defined with many species having now been described sharing a necromenic association with scarab beetles, a host they frequently share with other nematode species¹⁸. *P. pacificus* therefore provides an excellent model system with which to dissect the evolution of novel behaviors and their ecological significance.

In order to analyze predatory feeding behaviors in nematode species such as *P. pacificus* we developed several novel behavioral assays for easy observation and quantification of predatory actions. As *P. pacificus* displays a dimorphic mouth structure, which strongly influences predatory behavior, identification of the correct morphotype is essential^{1,2}. The narrow mouthed stenostomatous morph contains a single blunt dorsal tooth and does not engage in any predatory feeding. Alternatively, the wide mouthed eurystomatous morph includes a much larger claw shaped dorsal tooth and an additional opposing sub-ventral tooth, which together operate to efficiently open the cuticle of their prey. The ratio of the predatory eurystomatous to the non-predatory stenostomatous form varies among *Pristionchus* species and also within *P. pacificus*, however, the percentage of eurystomatous mouth morph in the *P. pacificus* wild type strain (PS312) is usually 70 - 90%². Additionally, the mouth form ratios can fluctuate depending on differing environmental influences (both known, including starvation and some small molecule signaling as well as unknown factors), thus correct identification and isolation of the predatory eurystomatous mouth form is essential for successful predatory assays.

Alongside the description of the predatory mouth form we have developed a "bite assay" for direct observation and quantification of predatory behaviors including biting, killing and feeding events. Here prey nematodes are isolated through filtering of newly starved cultures and exposed to predatory adult *P. pacificus*, which are observed together over a short time span. In addition, we have also developed a high throughput "corpse assay" to facilitate rapid screening of predatory behavior through indirect observation of predatory events. This takes advantage of the presence of larval corpses as a tool to screen for predation. Both assays provide easy and highly repeatable methods for observing and measuring predatory behavior in nematode species such as *P. pacificus*.

Protocol

1. Mouth form Phenotyping

1. Mouth Form Identification on Agarose Pads

Note: In order to visualize the nematode mouth morphs, immobilize the worms with a mild anesthetic treatment on agarose pads and observe as follows.

1. Grow and maintain nematode cultures such as *P. pacificus* on 6 cm standard nematode growth media (NGM) plates and feed on a bacterial lawn of *E. coli* OP50¹⁹.
2. Make agarose pads by first adding 0.06 g agarose to 3 ml H₂O in a 15 ml tube to make 3 ml 2% agarose solution. This can be stored for up to a year at 4 °C.
3. Mix and melt the agarose thoroughly in a microwave or alternatively use a heat block set to > 88 °C.
4. Once fully melted, add 10 µl of a 10 % sodium azide solution to the agarose and mix thoroughly. CAUTION: Dry sodium azide is reactive and all forms are toxic.
5. Using a 1 ml micropipette, place a drop of no less than 300 µl of the liquid agarose azide mix onto the middle of a standard glass microscope slide.
6. Before the agar cools, quickly place a second microscope slide on top of the drop in order to flatten the agarose which forms a pad upon cooling. Repeat for as many pads as is required.
7. Just prior to use, peel apart the glass microscope slides by sliding them off one another. Note: If agarose pads are prepared too far in advance they can be excessively dry and may damage the nematodes.
8. To transfer worms to the anesthetic agarose pads, place a drop of M9 buffer (2 - 3 µl) onto the center of the pad. Pick 2 - 3 young *P. pacificus* adults into the drop of M9 before placing a cover slip carefully over the pad. The *P. pacificus* nematodes will be immobilized in the agarose and ready to visualize.
9. Transfer the microscope slide containing the anesthetized worm to an appropriate microscope and observe under 63X Nomarski optics. Categorize morph identities based on the following features: the presence of an additional sub-ventral tooth, enlarged dorsal tooth and wide mouth opening is indicative of a eury stomatous mouth morph animal, while the presence of a single dorsal tooth and narrower mouth opening indicates a stenostomatous animal (**Figure 1**).
Note: In order to maintain the health of the animal, worms should be maintained on the agar pad for no more than 5 min.
10. After mouth morph identification, recover either eury stomatous or stenostomatous nematodes as required by removing the cover slip by gently sliding it off the agarose pad. Carefully pick the selected animals from the agarose pad (*E. coli* OP50 can be used on the pick to help make it stickier) onto fresh NGM plates. Allow recovery from the anesthetic until normal motile behavior has resumed upon which animals are ready for further predatory assays.

2. Rapid Mouth Phenotyping

Note: Alternatively, with more experience, mouth form type can be assayed without the need for any anesthetic treatment via a stereomicroscope with high magnification (150X).

1. Place nematodes on standard NGM plates with a bacterial lawn of *E. coli* OP50 onto the microscope viewing area.
2. Detect differences in mouth size and width. Note: At this magnification no tooth like structures are observable therefore mouth morph identification is solely based on wide mouths versus narrow mouths.

2. Bite Assay

Note: Biting assays permit a detailed predatory behavioral analysis.

1. Grow and maintain nematode cultures on standard NGM plates (6 cm) and feed on a bacterial lawn of *E. coli* OP50¹⁹.
2. Make assay plates by growing a large quantity of selected prey nematode larvae such as *C. elegans* or alternatively an appropriate ecologically relevant prey. Note: Adult *C. elegans* are too large to be suitable prey so it is important to use the larval stage.
 1. Maintain *C. elegans* or other potential prey species on standard NGM plates and feed on a bacterial lawn of *E. coli* OP50 until the population is freshly starved, resulting in an abundance of young L1 larvae.
Note: Time for starvation is dependent on numerous environmental and experimental factors, including number of nematodes used to start the culture, the amount of *E. coli* OP50 added, and the ambient temperature.
3. Wash four or more freshly starved prey plates with M9 and pass the worm solution through two 20 µm filters to remove all large animals and any remaining eggs before collection in a 15 ml tube. Only small larvae should remain in the solution.
4. To form a larval pellet centrifuge the filtered prey at 377 x g for 1 min.
5. Pipette 3 µl of pure worm pellet onto a 6 cm NGM plate with no *E. coli* OP50 present and wait at least 30 min for larvae to spread out sufficiently to generate an assay plate.
Note: 3 µl of pure worm pellet on the standard assay plates contain > 3,000 prey larvae. This is sufficient to generate frequent contact between predators and prey.
6. Screen predatory nematodes for the required mouth morph (protocol 1).

- Using standard worm picking techniques and a light stereomicroscope¹⁹, transfer correctly classified predators on to the assay plate. Take care to transfer as little OP50 bacteria as possible to the assay plate when transferring predators in order to minimize bacterial contamination. Wait 15 min to allow the worm to recover from the stress of being transferred and check for wild-type motile behavior to ensure worms have not been damaged from the transfer.
Note: There is no need to starve *P. pacificus*, as they are highly efficient predators of other nematode larvae even while well fed upon bacteria.
- After recovery, observe the predator using a light stereomicroscope for 10 min. With this equipment, observe and characterize distinct feeding events such as **biting**, characterized by the predator restricting the movement of the prey; **killing**, where by an opening of the prey cuticle is detected; and **feeding**, categorized by an observable consumption of the prey innards (**Figure 2A, B** and **Movie 1**).
- Repeat the assay by screening and observing a minimum of 10 individual predatory nematodes to ensure accuracy.

3. Corpse Assay

Note: Corpse assays facilitate a more rapid quantification of predatory behavior.

- Grow and maintain nematode cultures on standard NGM plates and feed on a bacterial lawn of *E. coli* OP50¹⁹. Generate triplicates of the assay plates mentioned previously (protocol 2.1 - 2.5).
- Screen predatory nematodes for the required mouth morph as described in protocol 1. Using standard worm picking techniques and a light stereomicroscope transfer 5 predatory nematodes with the required mouth morph to each assay plate. Leave the predators together with the prey for 2 hr.
- After 2 hr screen the assay plate for the presence of emptied corpses (**Figure 2B** and **C**). Identify corpses by the absence of motility along with obvious morphological defects including leaking innards or missing worm fragments.

4. Analysis of Pharyngeal and Tooth Movement

- Grow and maintain nematode cultures on standard NGM plates and feed on a bacterial lawn of *E. coli* OP50¹⁹. Generate assay plates as mentioned previously (protocol 2.1 - 2.5). If standard 6 cm NGM plates do not fit between the objective and the microscope stage, use the lid of small 35 mm petri dishes containing 2 ml NGM as a suitable alternative.
- Screen predatory nematodes for the required mouth morph as described in protocol 1. Using standard worm picking techniques and a light stereomicroscope, transfer a single correctly classified predator on to the assay plate. Wait 15 min to allow the worm to recover from the stress of being transferred.
- Observe predatory animals on a microscope at 40 - 63X Normaski, with a high-speed camera (**Movies 2** and **3**). Record pharyngeal pumping and tooth movement over 15 sec, at 50 Hz in at least 20 animals to ensure accurate quantification. Replay recorded movies at the desired speed in order to count individual pumps and tooth events.
Note: Pumping is observed in the corpus, located at the middle of the pharynx, while tooth movement is detectable in the mouth opening and is only observed from the dorsal tooth.

Representative Results

Following successful identification of the appropriate mouth morph in *P. pacificus*, clear differences between eury stomatous and stenostomatous animals can be detected (**Figure 3**) with only the eury stomatous animals engaging in killing behavior. In stenostomatous animals this behavior seems to be suppressed entirely. Furthermore, differences in the tooth activity and pharyngeal pumping of eury stomatous animals on bacteria and prey (**Figure 4** and **Movies 2** and **3**) are also evident. While predatory feeding, the pumping rate is reduced below that observed during bacterial feeding and tooth movement is detected in a one to one ratio with the pharyngeal pumping. This is potentially indicative of key regulatory mechanisms modulating the behavioral response to differing diet.

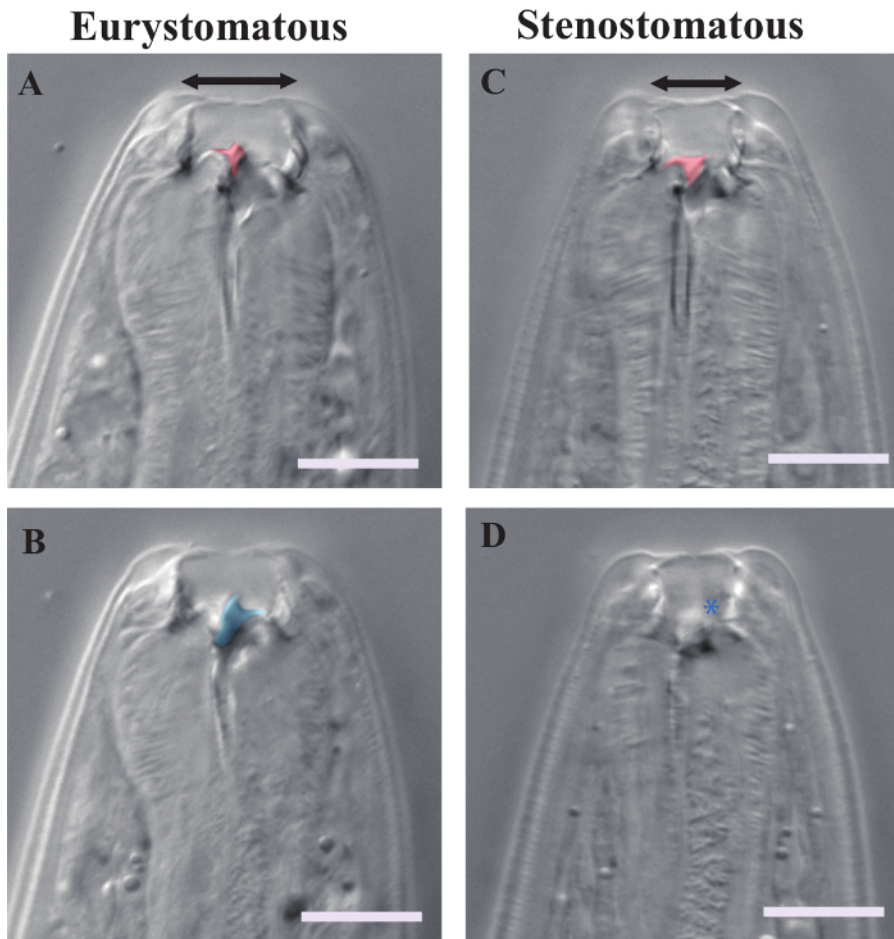


Figure 1. *P. pacificus* has a Mouth Dimorphism which Influences Feeding Behavior. (A) The eurystomatous mouth form is capable of predation and has a wide mouth opening with a large claw shaped dorsal tooth (false-colored red) and (B) a large opposing hook shaped sub-ventral tooth (false-colored blue). (C) The stenostomatous mouth form is only able to feed on bacteria and has a narrower mouth opening with a flint shaped dorsal tooth (false-colored red) and (D) no sub-ventral tooth (*). Normaski images are 63X and scale bar represents 10 μ m. [Please click here to view a larger version of this figure.](#)

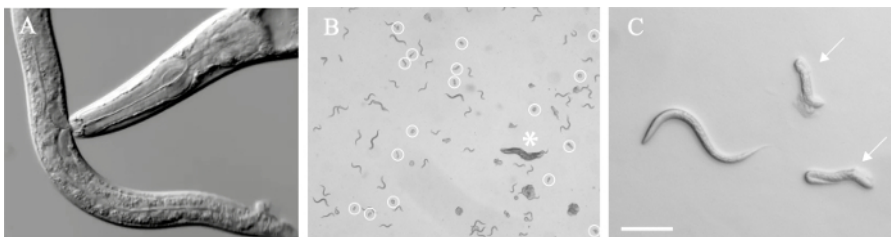


Figure 2. Predation Assays. (A) *P. pacificus* bites and kills the larvae of other nematodes such as *C. elegans*. (B) For biting assays, the number of bites by predators (*) can be observed using a light stereomicroscope and successful killing and feeding events also recorded. Corpses are also clearly visible (circles). (C) For corpse assays, larval carcasses (arrows) can be easily identified compared to living larvae. Scale bar represents 1 mm in B and 150 μ m in C. [Please click here to view a larger version of this figure.](#)

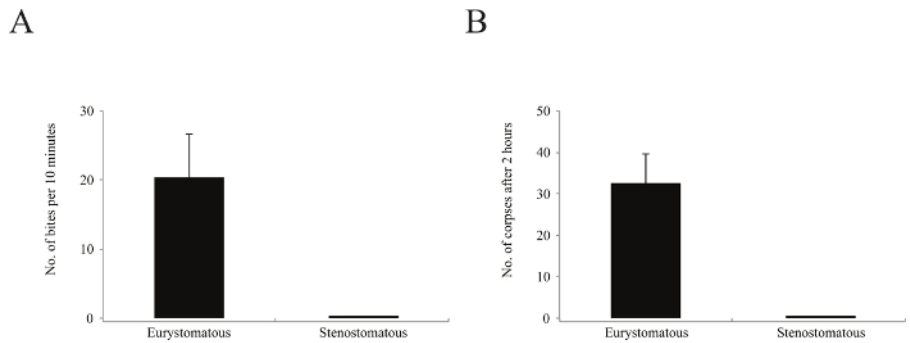


Figure 3. Results of Bite and Corpse Assays on *C. elegans* Prey. (A) Biting behavior is only evident in the eurystomatous mouth form with this behavior not displayed in stenostomatous animals. Error bar represents standard deviation of 10 replicates. (B) Coinciding with no biting behavior evident from stenostomatous animals, corpse assays also reveal carcasses only on assay plates of eurystomatous animals. Error bar represents standard deviation of 5 replicates. [Please click here to view a larger version of this figure.](#)

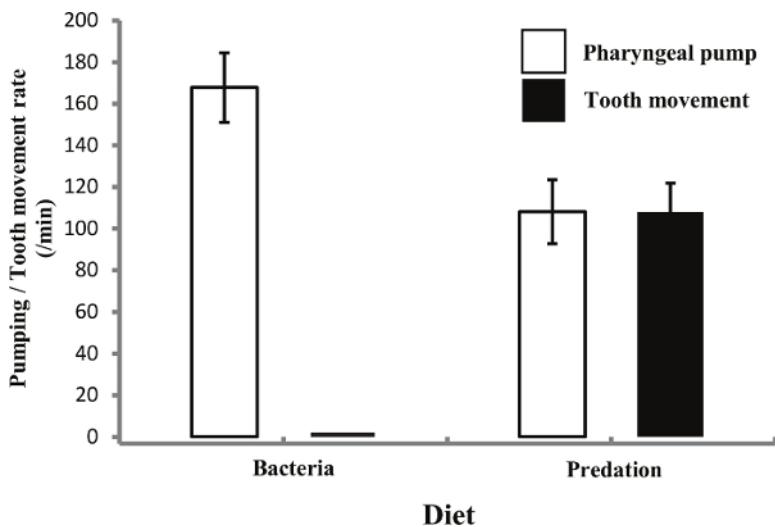
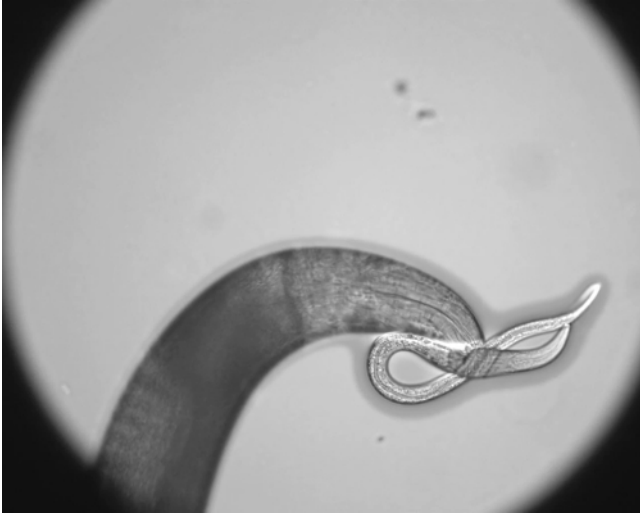


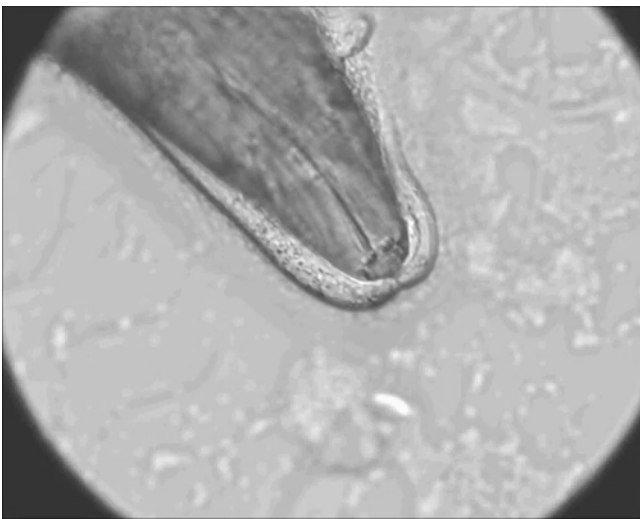
Figure 4. Eurystomatous Pumping Rate and Tooth Movement during Predatory Feeding. Tooth movement is only observed while eurystomatous animals are engaged in predatory feeding. This also coincides with a reduction in pharyngeal pumping. Error bar represents standard deviation of 10 replicates. [Please click here to view a larger version of this figure.](#)



Movie 1. Observation of Killing Behavior for Bite Assay using a Light Stereomicroscope. (Right click to download).



Movie 2. High-speed Camera Movie of *P. pacificus* killing *C. elegans* Larvae. (Right click to download).



Movie 3. Zoomed in High-speed Camera of Tooth Movement During Predation. (Right click to download).

Discussion

Nematodes provide a powerful system for understanding neurobiology and behavior with *C. elegans* thus far being the primary tool. However, numerous nematode species including *P. pacificus* display behaviors, which are absent or vary in complexities from the model organism *C. elegans* and therefore raise fascinating questions regarding the evolution and regulation of these behaviors. One such additional behavior found in many other nematode species including *P. pacificus* is the capacity to supplement their bacterial diet by engaging in predatory feeding^{1,20}. We have therefore developed and described a detailed protocol for easy and rapid characterization of these previously unanalyzed predatory behaviors in nematodes.

Firstly, we have provided methods to screen for variations in feeding apparatus within the nematode mouth. The identification of the correct mouth type is an essential first step for successful predation assays as, at least within the *Pristionchus* genus only eury stomatous animals are capable of predatory feeding. It is best to identify mouth morphs with the "rapid mouth phenotyping" protocol described in protocol 1.2 as this method is much less invasive and therefore it is less likely that predatory behaviors may be perturbed. However, it is recommended to first become familiar with the different mouth structures by identification with anesthetized animals on agar pads (protocol 1.1).

Following identification of the desired mouth morph, we have described two assays for quantifying predatory feeding. These are a rapid, high throughput "corpse assay" (protocol 3) and a more time consuming but more in-depth behavioral analysis through the "bite assay" (protocol 2). Both of these protocols are highly flexible allowing for several modifications in order to optimize the assays depending on the experimental requirements. For bite assays using *P. pacificus* predators on *C. elegans* prey, observations of predatory behavioral interactions for a time window of 10 min was sufficient to quantify a significant amount of bites along with other feeding events. For "corpse assays" again utilizing *P. pacificus* predators on *C. elegans* prey, 5 predators for 2 hr produced easily quantifiable and consistent corpse numbers allowing for rapid behavioral analysis. However, it should be noted different species of predatory nematode move at different speeds, eat at different rates and generally demonstrate a large diversity in other behaviors¹. Additionally, different prey species may also be eaten at different rates for similar reasons. It is therefore recommended to optimize the assays based upon the nematode species tested both as predators and prey, and also for any differences in environmental conditions. During both "bite" and "corpse" assays it is critical that both prey and predators are healthy,

as stressed or injured predators will not kill efficiently. In addition, fresh assay plates are essential as older plates can become dried out which adversely affects the health of the nematodes leading to erroneous assays. It is also hoped that future iterations of these predatory assays will be able to take advantage of recent advances in technology in order to automate much of the analysis as has been accomplished for investigating many behaviors observed in *C. elegans*^{21,22}. Currently problems are likely to arise in nematodes such as *P. pacificus* as they appear much more sensitive to contact, making isolation and immobilization in microfluidic chambers likely to abrogate predatory feeding. Overcoming this may prove challenging but would facilitate individual nematodes to be screened for subtler predatory behaviors.

Finally, we have also provided methods for examining the nematode feeding apparatus itself facilitating comparisons between predatory and bacterial feeding modes by quantifying the tooth and pharyngeal pumping kinetics using a high-speed camera (protocol 4). The quantification of pharyngeal pumping rates in *C. elegans* has been utilized to monitor feeding for many years²³, however, *C. elegans* lacks any form of mouth denticle and also lacks predatory behaviors. Through combining the quantification of pharyngeal pumping with that of tooth activity, any innervation of the teeth specific to predation can be also observed. Due to the magnification required to observe the tooth movement the animals often move out of the focal plane, thus it is usually only possible to observe the tooth for short time windows. Additionally, unlike *C. elegans*, the pharynx of *P. pacificus* does not continuously pump, rather it engages in spells of pumping and feeding. Therefore, for accurate pharyngeal pumping rates while feeding to be determined, it is important to record 15 sec of continuous feeding.

These methods presented here therefore provide the first framework for investigating predatory behaviors in nematode systems. Moreover, they may also be adaptable for use in investigating other interactions within the nematode ecosystem including the influence of additionally ecologically relevant organisms on predation including microorganisms, fungi and mites. Thus they provide a means to dissect how these predatory behaviors are regulated, how they may have evolved and also their ecological significance.

Disclosures

The authors declare no financial or competing interests.

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