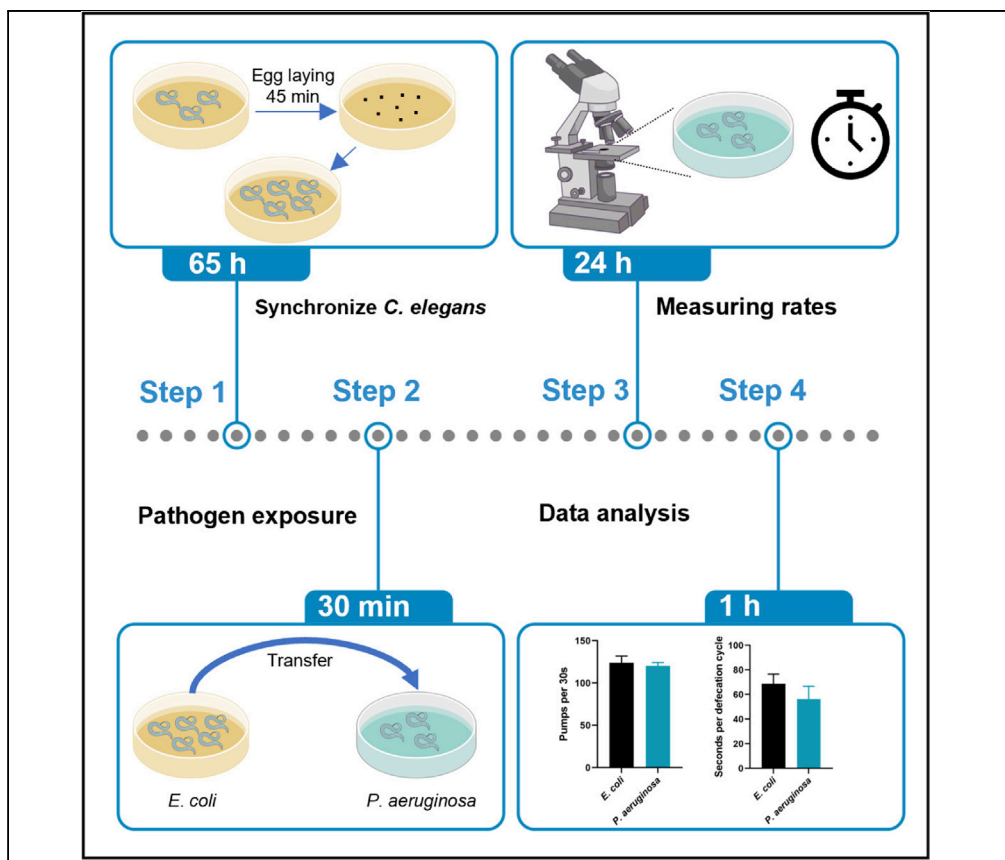# STAR Protocols

Protocol

Protocol to measure bacterial intake and gut clearance of *Caenorhabditis elegans*

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Highlights
Measuring changes in
feeding and
defecation rates in *C.
elegans*

Protocol for exposing
synchronized *C.
elegans* to
pathogenic bacteria

Analysis of feeding
and defecation rates

The nematode *Caenorhabditis elegans* is a common model organism for studies in immunity and host-pathogen interactions. In this protocol, we describe techniques for measuring pharyngeal pumping and defecation rates of adult *C. elegans* exposed to either pathogenic or non-pathogenic bacteria. Quantifying changes in pumping or defecation rates during pathogenic infection can account for behavioral changes that may affect survival. We also detail how to synchronize *C. elegans* and expose the synchronize animals to either pathogenic or non-pathogenic bacteria.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Protocol

Protocol to measure bacterial intake and gut clearance of *Caenorhabditis elegans*

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https://doi.org/10.1016/j.xpro.2022.101558

SUMMARY

The nematode *Caenorhabditis elegans* is a common model organism for studies in immunity and host-pathogen interactions. In this protocol, we describe techniques for measuring pharyngeal pumping and defecation rates of adult *C. elegans* exposed to either pathogenic or non-pathogenic bacteria. Quantifying changes in pumping or defecation rates during pathogenic infection can account for behavioral changes that may affect survival. We also detail how to synchronize *C. elegans* and expose the synchronize animals to either pathogenic or non-pathogenic bacteria.

For complete details on the use and execution of this protocol, please refer to Wibisono et al. (2022).

BEFORE YOU BEGIN

The pathogenic bacterium described in this protocol is classified as a BSL-2 organism and as such this protocol should only be performed in a BSL-2 laboratory. Prior to beginning these methods, all animals should be well-fed throughout maintenance (Baugh and Hu, 2020). Starvation should be avoided as it leads to transgenerational changes in behavior (Jobson et al., 2015).

Institutional permissions (if applicable)

Experiments involving *C. elegans* do not require IACUC approval.

Media preparations

⏱ Timing: 1 week

In the following sections, the media and buffers required to measure the pumping and defecation rates will be prepared. The amount of media made in this section is enough to perform at least three biological replicates for both pumping and defecation rate measurements.

1. Prepare sterile lysogeny broth (LB) agar plates and liquid media following the manufacturer's instructions.
2. Streak *Escherichia coli* (*E. coli*) strain OP50 on a LB agar plate. Incubate the bacteria streak at 37°C for 16 h.
3. Store the *E. coli* OP50 streak plate at 4°C after incubation. The *E. coli* OP50 plate can safely be stored at 4°C for one week if wrapped in parafilm to prevent moisture loss.
4. Refer to the included recipe and prepare two 1 L batches of Nematode Growth Media (NGM). Autoclave the media for 30 min at 121°C.

Note: Autoclave 1 M CaCl$_2$, 1 M KPO$_4$ (pH6.0), and 1 M MgSO$_4$ in separate containers at 121°C for 15 min. Filter sterilizes 5 mg/mL of cholesterol through a 0.22 μm filter after being dissolved in 95% ethanol. Prepare all previously mentioned reagents prior to autoclaving the NGM.

Note: 1 M CaCl$_2$, 1 M KPO$_4$ (pH6.0), 1 M MgSO$_4$, and 5 mg/mL cholesterol can be stored at 20°C for up to three months.

5. After autoclaving, allow the media to cool to 50°C in a water bath before adding the CaCl$_2$, KPO$_4$, MgSO$_4$, cholesterol to the media, pour 1 L of the NGM into 100 60 mm Petri dishes. Each 60 mm dish requires approximately 10 mL to fill. Pour the remaining 1 L of NGM into 240 35 mm Petri dishes. Each 35 mm dish requires approximately 4 mL to fill. Let NGM plates cool on bench 16 h at 20°C.

Note: Add the post-autoclave reagents to each liter of media just before pouring.

6. Inoculate two 50 mL conical tubes containing 13 mL of sterile LB with *E. coli* OP50 from the previous streak plate. Incubate the broth at 37°C for 24 h in a shaking incubator maintained at 200 rpm.

Note: For consistency, inoculate both tubes with the same single colony from the streak plate. If this proves difficult, inoculate a 50 mL conical tube with 5 mL of sterile LB with a single colony and incubate the culture for 16 h at 37°C in a shaking incubator maintained at 200 rpm the day before preparing the NGM plates. Store the fresh liquid culture at 4°C for up to two days. Inoculate the two 13 mL of broth with 25 μL of liquid culture and incubate under the same conditions as mentioned above.

7. Seed the fresh 60 mm NGM plates with 200 μL of the fresh *E. coli* OP50 liquid culture and spread the liquid culture by gently swirling the plate. Allow the *E. coli* OP50 to grow at 20°C for 72–96 h or until a visibly thick lawn appears.

Note: After 24 h, move the freshly seeded NGM plates to a covered container to reduce moisture loss and extend the life of the plates.

Note: Seed NGM plates can be stored in at 20°C for 3 weeks.

8. Place the unseeded 35 mm NGM plates in a container and move them to the 4°C for storage. The NGM plates can be stored in the 4.

⚠ CRITICAL: NGM plates can be stored at 4°C for approximately 1 month.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Escherichia coli OP50	CGC	N/A
Pseudomonas aeruginosa PA14	Bei Resources	NR-50573
Chemicals, peptides, and recombinant proteins		
Bacto peptone	VWR International	90000-264
Sodium Chloride	VWR International	BDH9286-12KG
		(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Criterion Agar	VWR International	C7432
Calcium dichloride	MilliporeSigma	C4901
Magnesium Sulfate	MilliporeSigma	M7506
Potassium phosphate dibasic	MilliporeSigma	P3786
Potassium phosphate monobasic	MilliporeSigma	P0662
LB Broth	MilliporeSigma	L3022
Cholesterol	MilliporeSigma	C8667
100% absolute molecular grade ethanol	Fisher Scientific	BP2818
Experimental models: Organisms/strains		
Caenorhabditis elegans wild-type (N2)	CGC	N/A
Other		
60 mm Petri dish	Tritech Research	T3315
35 mm Petri dish	Tritech Research	T3500
50 mL conical centrifuge tubes	Thermo Fisher Scientific	339652
Stericup 500 mL Millipore Express Plus 0.22 μm PES	MilliporeSigma	SCGPU10RE
Leica M80 stereomicroscope	Leica Microsystems	N/A
Autoclave	Sanyo	N/A
37°C incubator	Thermo Fisher Scientific	Forma 370
25°C incubator	Thermo Fisher Scientific	Forma 3920
Isotemp water bath	Fisher Scientific	FSGPD10
Shaking incubator MaxQ	Thermo Fisher Scientific	SHKE4000
Advance magnetic stirrer	VWR	76447-052
Fisherbrand™ Traceable™ Four-Channel Countdown Alarm Digital Timer/Stopwatch with Memory Recall	Fisher Scientific	14-649-17
GraphPad Prism	GraphPad	www.graphpad.com

MATERIALS AND EQUIPMENT

1 Liter of Nematode Growth Media		
Reagent	Final concentration	Amount
NaCl	51 mM	3 g
Agar	n/a	17 g
Bacto Peptone	n/a	2.5 g
ddH_2O	n/a	975 mL
$MgSO_4$ (1 M)	1 mM	1 mL
$CaCl_2$ (1 M)	1 mM	1 mL
Cholesterol (5 mg/mL)	5 μg/mL	1 mL
KPO_4 pH 6.0 (1 M)	25 mM	25 mL

The following media is required for this protocol (see also (Stiernagle, 2006)).

Add the $MgSO_4$, $CaCl_2$, Cholesterol, and KPO_4 to the NGM after letting the media cool to 50°C in a water bath. Store the cooled plates at 4°C for up to 1 month.

⚠ CRITICAL: Do not add the $MgSO_4$, $CaCl_2$, Cholesterol, or KPO_4 before the NGM cools to 50°C as it will cause the reagents to precipitate out of the media and form crystals.

STEP-BY-STEP METHOD DETAILS
Experiment preparations

⏱ Timing: 1 week

In this section, wild-type *C. elegans* will be synchronized via timed egg laying. *E. coli* OP50 and *P. aeruginosa* PA14 NGM plates will be prepared for measuring pumping and defecation rates.

1. Transfer 5 well-fed wild-type gravid adult *C. elegans* to a fresh maintenance NGM plates seeded with *E. coli* OP50.
2. Allow the animals to lay eggs and for the progeny to grow at 20°C until they reach gravid adult stage. The new eggs will develop into gravid adult animals in approximately 96 h.

    Note: Monitor the amount of bacteria on the lawn as the animals develop. Avoid starving the animals by transferring them to fresh plates if the bacteria appear depleted.

3. Synchronize the animals by transferring 20 gravid adults to a NGM plate seeded with *E. coli* OP50.
4. Place the plate with the 20 gravid adults into a 25°C incubator for 45 min to lay eggs.
5. After 45 min, retrieve the plates from the 25°C and remove all the gravid adults from the plates. Place the plates with the newly laid eggs in the 20°C for 65 h.

    Note: Count the number of adults as they are removed from the plate to ensure that all 20 adult animals have been removed. If an adult is left on the plate, it will ruin the synchronization and delay the experiment.

6. Streak fresh *E. coli* OP50 on a new LB agar plate on the same day of animal synchronization and incubate the plate at 37°C for 16 h.
7. Streak fresh *Pseudomonas aeruginosa* PA14 on a new LB agar plate on the same day of animal synchronization and incubate the plate at 37°C for 16 h.
8. The following day, inoculate 5 mL of liquid LB with a single colony from the new *E. coli* OP50 streak plate, inoculate another 5 mL of liquid LB with a single colony from the new *P. aeruginosa* PA14 streak plate. Incubate both broths for 16 h in a shaking incubator at 200 rpm.
9. The next day, seed five 35 mm NGM plates with 30 μL for both *E. coli* OP50 and *P. aeruginosa* PA14 broth for a total of ten plates and incubate at 37°C for 16 h.
10. Retrieve the ten 35 mm NGM plates from the 37°C the next day and allow the plates to cool on the bench for 30 min.

Pumping rate quantification

⏱ Timing: 2 days

In this section, synchronized *C. elegans* will be exposed to both *E. coli* OP50 and *P. aeruginosa* PA14. The pumping rates of individual animals on both bacterial conditions will be measured three times in 30 s intervals to create a scatterplot and average pumping rate.

11. Transfer ten 65 h old, synchronized adult animals to each of the fresh 35 mm NGM plates seeded with *E. coli* OP50 or *P. aeruginosa* PA14.
12. Place the ten 35 mm NGM plates with 65 h old animals in a 25°C incubator for 24 h.
13. The next day, retrieve the animals from the 25°C incubator.
14. Set a timer for 30 s before finding an animal under a Leica M80 dissecting stereomicroscope.
15. Focus on the terminal bulb of the pharyngeal grinding. Refer to Figure 1 for the location of the terminal bulb.
16. Start the timer and begin counting the complete cycles of the terminal bulb for the full 30 s.

    Note: A complete cycle of the terminal bulb is defined as the up and down motion of grinder opening and closing as bacteria enter (Avery, 1993).

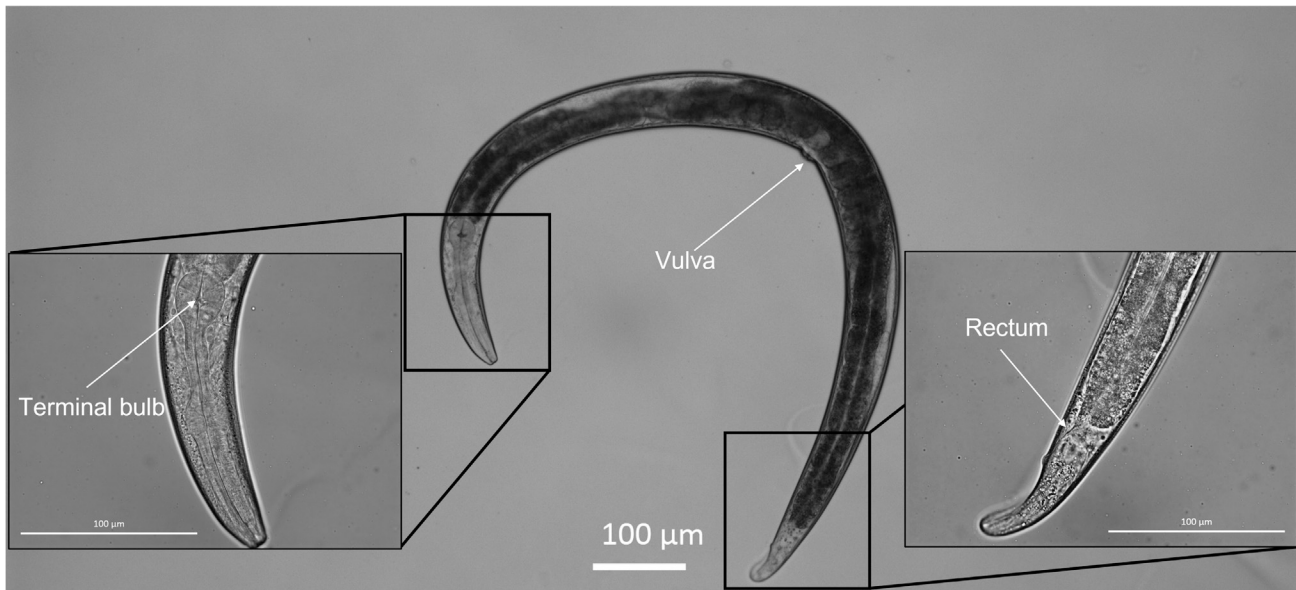**Figure 1. Labeled DIC image of an adult *C. elegans***

Note: The animal will move through the bacteria as they eat. Be sure to follow the animal on the microscope as you count and do not try to touch the animal before counting the pharyngeal pumping rate. Touching the animals with the pick will increase their pumping rate temporarily and skew your results.

17. Record the number of pharyngeal contractions and continue counting the same animal's pumping rate two more times.
18. After counting the pumping rate in triplicate, remove the animal from the plate.
19. Count and record a total of 10 individual animals per bacterial conditions.
20. Average the pumping rate for each individual animal.
21. Plot the averages for each individual animal in GraphPad Prism to find the average pumping rate and standard deviation for the whole group.

Defecation rate quantification

⏱ Timing: 2 days

In this section, synchronized *C. elegans* will be exposed to both *E. coli* OP50 and *P. aeruginosa* PA14. The defecation rates of individual animals on both bacterial conditions will be measured five times by measuring the time between rectal muscle contractions to create a scatterplot and average defecation rate.

22. Transfer ten 65 h old, synchronized adult animals to each of the fresh 35 mm NGM plates seeded with *E. coli* OP50 or *P. aeruginosa* PA14.
23. Place the ten 35 mm NGM plates with 65 h old animals in a 25°C incubator for 24 h.
24. The next day, retrieve the animals from the 25°C incubator.
25. Focus on the rectum of an individual animal and track the animal under the microscope. Refer to Figure 1 for the location of the rectum.
26. Using a stopwatch, start the watch at the first intestinal expulsion. Follow the animal and press the stopwatch's lap button on the next intestinal expulsion.

Note: Defecation or expulsion of the intestinal contents can be seen as both a contraction of the rectal muscles near the tail of the animals or a visible ejection of material from the nematodes.

Note: The animal will move through the bacteria as they eat. Be sure to follow the animal on the microscope as you count and do not try to touch the animal before counting the defecation rate. Touching the animal will increase pumping rate and in turn may affect the defecation rate.

27. Continue measuring the time between defecations four more times for a total of five measurements.
28. After measuring the defecation five times, remove the animal from the plate.
29. Count and record a total of 10 individual animals per bacterial condition.
30. Average the defecation times for each individual animal.
31. Plot the averages for each individual animal in GraphPad Prism to find the average defecation rate and standard deviation for the whole group.

EXPECTED OUTCOMES

The wild-type animals did not have a significantly different pumping rate between feeding on *E. coli* OP50 and *P. aeruginosa* PA14, however they did have a significantly shorter defecation time on *P. aeruginosa* PA14 compared to *E. coli* OP50. The significance for both experiments was determined using a two-tailed Student's t-test.

Figure 2.

This protocol is presented as a framework for measuring two of the behavioral responses *C. elegans* utilize to defend itself during pathogen infection. Changing the pathogen or strain of *E. coli* that the animals are maintained on, or measuring mutant animals may change the results of the experiment.

LIMITATIONS

This protocol describes how to quantify the feeding behavior of *C. elegans* in both pathogenic and non-pathogenic conditions. *C. elegans,* however, are extremely sensitive and any contamination before the experiment can alter the animal's behavior response. It is key that the media, bacterial conditions, and animal age are consistent between replicates.

TROUBLESHOOTING
Problem 1
Difficulty synchronizing animals (step 3).

Potential solution
Smaller larval animals can accidentally be picked along with the intended gravid adults. To reduce the chance of picking larval worms, pick the intended gravid adult animals and move them to a fresh NGM plate seeded with *E. coli*. Allow the animals to crawl and spread out on the bacterial lawn before picking the adults for synchronization.

Problem 2
Significant loss of animals due to matricide or desiccation (steps 13 and 24).
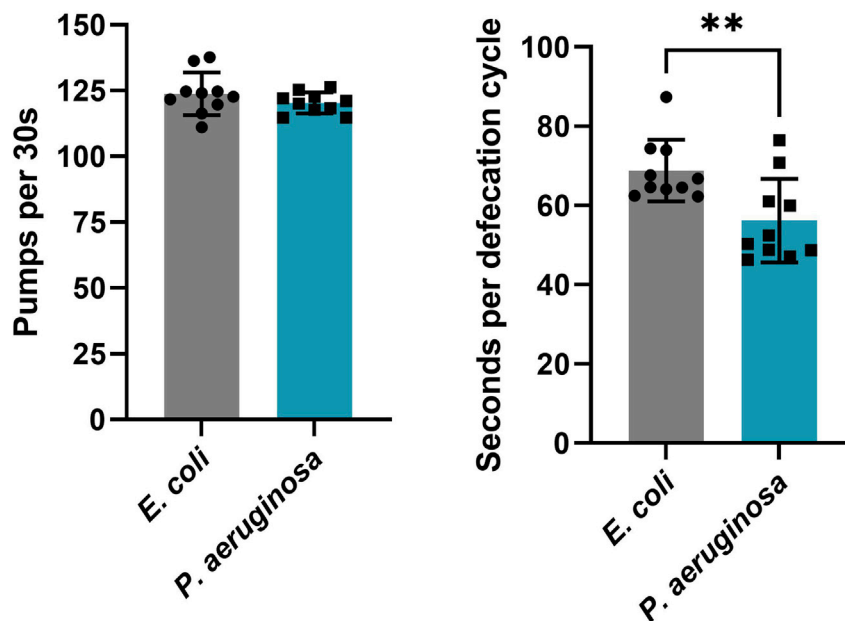
Figure 2. *P. aeruginosa* infection affects defecation rate but not pumping rate in wild-type *C. elegans*
(A and B) Synchronized wild-type animals were exposed to *E. coli* OP50 or *P. aeruginosa* PA14 for 24 h before pumping rates (A) and defecation rates (B) were measured. (A) $p = 0.2249$. (B) $p = 0.0070$. Error bars indicated SD.

Potential solution

C. elegans will strongly avoid certain pathogenic bacteria such as *P. aeruginosa.* If there is a significant loss of animals to either matricide (eggs hatching while still inside the animal) or desiccation on the walls of the Petri dish, use more plates and animals than originally suggested.

Problem 3

Difficulty transferring animals from one plate to another (steps 1, 3, 5, 11 and 22).

Potential solution

An improperly prepared worm pick can make transfer animals difficult. The pick should be bent to resemble a hockey stick and the end flattened to increase surface area (Stiernagle, 2006). Fresh NGM plates can also making picking animals difficult as the presence of excessive moisture can make the *E. coli* OP50 blob at the end of the pick soft and watery. A solution to watery *E. coli* OP50 is to collect a blob of bacteria from an unused plate then quickly touch the end of the pick to a flame to dry the bacteria blob. With the dried bacteria still attached to the pick, collect another blob of bacteria. The heat-dried bacteria will draw moisture from the fresh bacteria causing the blob to become tacky and easier to pick animals with.

Problem 4

Mold or other contamination on NGM maintenance plates (step 1).

Potential solution

The nematode growth media can support a variety of microorganisms and it common for mold and other airborne organisms to begin growing on older NGM plates. If a contamination is spotted on a plate with *C. elegans*, the animals can be cleaned following the method outline in wormbook using sodium hypochlorite and sodium hydroxide (Stiernagle, 2006).

Problem 5

Pathogen contamination on control plates (steps 13 and 24).

Potential solution

P. aeruginosa PA14 will give the NGM plates a blueish hue. If the *E. coli* OP50 control animal plates after the 24 h incubation time show this change in color your control animals have been exposed to *P. aeruginosa* PA14 and the experiment cannot proceed. To prevent this, a pick specifically for picking animals to P. aeruginosa can be made and kept separate from the pick used for transferring animals from one *E. coli* OP50 plate to another.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jingru Sun (Jingru.sun@wsu.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate data or code.

AUTHOR CONTRIBUTIONS

Conceptualization, P.W. and J.S.; Methodology, P.W. and J.S.; Writing, P.W.; Review and editing, J.S.; Supervision, J.S.; Funding Acquisition, J.S.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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