nature portfolio

Peer Review File

FLP-15 functions through the GPCR NPR-3 to regulate local and global search behaviours in Caenorhabditis elegans

Corresponding Author: Dr Kavita Babu

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Attachments originally included by the reviewers as part of their assessment can be found at the end of this file.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Please see our formatted review attached
[pasted below for convenience]
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Review summary
We find this to be a solid and interesting paper. It claims that the transition from local to global search in C. elegans is regulated by FLP-15 through the I2 pharyngeal neuron via the NPR-3 receptor. These claims appear to be well supported by the data presented, though we would like to note that we are experts in C. elegans behavior, so our review focuses on the behavioral experiments and on the logic when interpreting the results from the different mutants; we are not specialists in neurobiology or genetics. From our point of view, the paper is adequate for publication in this journal, and we mainly offer minor suggestions that we hope may improve the paper's clarity.

MAJOR SUGGESTIONS

1. The main claim of the paper refers to the transition between local and global search, and the paper uses the frequency of a specific type of reversal (>2 head swings) as a proxy of the type of search. While this is a very reasonable proxy and all results are consistent with what we know about local/global search in C. elegans, the claim would be strengthened by a control showing that this proxy is indeed a good indicator of the type of search. To do this, the authors could show for example the Mean Squared Displacement in the two relevant periods (0-5 min and 25-30 min), and show that it correlates with the frequency of reversals. Including this analysis for both wild-type and mutant strains would further reinforce their argument.

2. The terms "local" and "global" search are used throughout the manuscript, including the Methods and Results sections, in a way that we fear may be misleading. In some places the authors seem to use "local search" as a synonym of what happens in the 0-5 min period and "global search" as a synonym of what happens in the 25-30 min period, even when the worm's behavior does not actually change (for example, lines 272-275). The terms "local search" and "global search" should be reserved for the cases where C. elegans actually shows these movement patterns, so we suggest reporting results using objective time intervals (e.g., 0–5 min and 20–25 min) and discussing local/global search in the Discussion, rather than implying direct measurement of the phenomenon in the Methods or Results.

3. Line 273: The authors state that the flp-15 mutant failed to reduce reversal frequency when transitioning from local to global search. However, according to Figure 2A, the issue seems not to be a failure to reduce, but rather a failure to exhibit the high initial reversal frequency observed in the wild type. The mutants maintain a constant low reversal frequency throughout the assay, suggesting that the local-to-global search transition might be absent in these animals.

4. Lines 340-341: Similarly, the authors state that the npr-3 mutants failed to exhibit a high reversal frequency during the local search phase, which raises the question of how comparable this behavior is to what is typically defined as local

search. In addition, the authors report that these mutants did not show a temporal decrease in reversal frequency during the "transition from local to global search," which could be confusing given that the Introduction describes this transition as being primarily driven by a decrease in reversal frequency.

MINOR SUGGESTIONS

Reversal quantification:
The authors quantify reversals using events longer than two head swings; however, it is unclear how representative these are of the total number of reversals executed by the animals. How was this threshold chosen? How representative are two-head-swing reversals, relative to all reversal events?

Mapping figures to experiments:
It would help readers if a clearer connection between figures and the corresponding experiments in the Methods section were provided, for example by specifying whether it was a single tracking experiment or a multi animal tracking experiment. Currently, it is difficult to determine which experimental data corresponds to each figure (for example, in Figure 2 it is not clear if data come from single animal experiments or not).

Figure color scheme:
Consider using a consistent color scheme across all figures. The current use of colors is sometimes confusing, as the same color may represent different aspects in different figures. For example, in Figure 2A gray/blue represent different time intervals but in Figure 2C they represent different strains. It would greatly improve readability to have a more consistent color scheme.

Figure scale:
Wherever possible, especially when displaying results for the 0–5 min and 20–25 min intervals in parallel plots, please use identical axis scales to facilitate visual comparison (For example, Figure 2C,D).

Statistical significance:
It would be helpful to clarify how statistical significance is represented in the figures. For example, in Figure 4 it is unclear what the asterisks on top of individual boxes mean. Is it significance with respect to WT? If so, this should be explicitly explained in the figure caption.

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More Specific comments / suggestions for the Authors

Abstract
•Line 34: The phrase "in C. elegans" seems unnecessary given that in previous line authors already specified "in C. elegans".
•Line 35: Please clarify what is meant by "cumulative reorientations" and how these can decrease if they are cumulative.

Introduction
•Line 80: Please either clarify what is meant by the "stereotypic locomotion of C. elegans" or consider omitting the term stereotypic.
•Line 90: This sentence repeats information already mentioned in line 78.
•Lines 96-99: A citation is needed to support this claim. These vertebrate-specific peptides do not exist in C. elegans, which may confuse readers. In this species, roaming and dwelling behaviors are mainly modulated by serotonergic and dopaminergic signaling.
•Lines 101–103: This passage is hard to follow; please rephrase for clarity.

Methods
•Line 132: Specify the age of the worms or clearly define what is meant by "young adult".
•Line 165: Consider omitting one of the "single" as there are two of them very close to each other.
•Line 170: Consider tone-down the sentence as it may not be possible to eliminate all bacteria within a 2-minute crawling time window.
•Line 171: The rationale for omitting cholesterol from the NGM plates should be clarified, since cholesterol can influence C. elegans physiology and behavior. Please explain why cholesterol-free media were used in experimental plates.
•Line 175: C. elegans should be italicized.
•Line 178: The phrase "under similar conditions" is ambiguous. Please clarify what aspects were kept constant between the initial and final recordings (e.g., same plate, temperature, illumination, and handling). Was the plate moved from the recorder between the first and the same period, or was it recorded continuously? This will ensure reproducibility and avoid confusion about potential manipulations during the 25-minute exploratory period.
•Line 179: Possible incorrect reference to Figure S2A.
•Line 184: Consider citing Chiba, C. M., & Rankin, C. H. (1990) to clarify what is meant by a "spontaneous reversal".
•Line 191: As mentioned above, the rationale for omitting cholesterol from the NGM plates and replacing agar with agarose should be clarified, as both modifications could potentially influence worm physiology and behavior, including pumping rate.
•Line 193: As mentioned above, the authors should clarify what "young adults" refers to.

•Line 195: How long did the starvation period last? In case it is 1 minute consider a more appropriate term other than starvation, which is often reserved for longer periods.

Results
•Line 255: consider in text citation of Figure S1A.
•Lines 274-276: This passage is hard to follow as local and global search are not conditions.

Discussion
•Lines 457-461: The authors present as two distinct results the observations that flp-15 mutants do not exhibit a high reversal frequency at the beginning of the experiment and that their reversal rate does not decrease over time. However, these two findings appear to describe the same phenomenon, since a reduction in an already low rate cannot be meaningfully detected. We suggest rephrasing this section (and any related statements elsewhere) to avoid presenting these as independent results.
•Lines 524-525: Please note that dwelling and roaming are behavioral states typically defined for on-food conditions and should not be conflated with off-food foraging or food-seeking patterns.

Figures
•Figure 1: It is a bit confusing that violin plots go below zero, given that a negative number of reversals cannot occur.
•Figure 2: i) The authors refer to a transition from local to global search even though flp-15 does not show a decay in reversal frequency. This could be misleading, as such a transition may not occur in this strain. ii) Including, an explicit spatial coverage metric for the first 5 minutes and the last 25 minutes would substantially strengthen the argument. iii) Caption (B): The statement that there is “no difference” between on food and off food conditions seems inconsistent with the data, which show a visible difference.
•Figure 3: i) The figure shows four boxplots, but the caption describes only three treatments (wild type, flp-15, and the transgene). Please clarify this discrepancy. ii) It is also unclear whether these experiments were performed individually or collectively; please specify.
•Figure S2B: “speed” would be more accurate than “velocity” on the axis (since “velocity” usually refers to a vector).
•Figure 4: i) The mutants appear to maintain similar reversal frequencies during the first 5 and the last 25 minutes of the experiments. Thus, describing this as a “transition from local to global search” may not be appropriate, since the data do not clearly support such a transition. ii) On line 782, after “background” there appears to be a typo as “in” should likely read “is”. Consider removing the phrase “in these mutants,” as it is redundant.
•Figure 6: Please clarify why the term dwelling is used here. Typically, dwelling and roaming refer to on-food behaviors and are defined based on locomotion speed rather than reorientations.

Reviewer #2

(Remarks to the Author)
Animals use a variety of sensory cues to explore their environment and direct them towards food. C. elegans undergoes a stereotyped behavior in the presence and absence of food. In particular, in the absence of food, animals progress from dwelling in its local environment to exploring a larger area, a behavior called foraging, to find food. Previous work from this group and others have shown that FLP-15 peptides, which are released from the proprioceptive pharyngeal I2 neurons, signal through the NPR-3 receptor in dopaminergic neurons to affect locomotory behavior. flp-15 mutants have an increased amplitude and increased number of body bends during reversal compared to wild-type animals. However, it was unclear from these authors’ previous paper (Bhat et al., 2025) whether what was scored as increased reversal length actually corresponded to an actual increase in the length of the reversal (as opposed to the number of head bends).

In this paper, the authors examine a switch from searching locally to globally for food during food deprivation. The authors look at the number of reversals, as opposed to number of body bends/reversals that was counted in their previous paper, made during searching. flp-15 mutants have a decreased reversal rate compared to wild-type animals in the absence of food. As on food, FLP-15 signals through the NPR-3 receptor expressed in dopaminergic neurons for this behavior. However, the authors did not indicate whether the number of body bends/reversal or distance traveled were affected. In addition, the number of reversals in flp-15 mutants do not appear to change over time without food. If the animals were recorded during the entire time, did flp-15 mutants show any change of behavior from 0-30 minutes?

A novel finding was that levels of flp-15 mRNA increase the longer an animal is under starvation conditions, leading the authors to propose that off food, the levels of flp-15 mRNA increase as the levels of dopamine decrease. Does this hypothesis extend to when animals are returned to food? Would flp-15 mRNA levels return to basal levels? In addition, how dopamine levels will be decreased in response to increased FLP-15 production and the mechanism for this compensation is unclear.

Overall, the paper has some interesting new work, but the hypotheses need to be fleshed out better and more results included. For instance, I2 is a proprioceptive neuron that senses oxidative stress and light in the pharynx to inhibit feeding. Because pharyngeal rates are not affected in flp-15 mutants, can I2 neurons also be sensors for food volume (i.e., the neuron monitors how much the pharynx is distended with food) to regulate movement? What input is being received by I2 neurons to activate ADE/CEP neurons?

Minor comments:
1. The alleles of the strains examined in depth should be included in the Materials & Methods. Only one allele of flp-15 and npr-3 were tested. At least two alleles of each gene should be tested, especially as at least one other allele of npr-3 is

available in the authors' lab.
2. Fig. 2B: Are the off food animals ones that were on food and then switched to off food? Or completely different animals?
3. Supplementary Information, Table S1: all alleles should be italized.
4. Lines 283, 307: Not supplementing flp-15, because it sounds like an additive to the petri dish. Transforming? Carrying the transgene?
5. For qPCR experiments indicate the housekeeping genes that were used for internal controls.
6. flp-15 expression in I2 has been previously reported and should be cited.
7. Supplementary figure legends Line 51: Please indicate to what the arrow heads are pointing.
8. Fig. 5 legend. Please indicate orientation of the animal (i.e., anterior is to the left? Ventral view?).
9. Fig. 6C: Reverse the order of the bars. Minus dopamine should be before no dopamine.
10. Fig. 7: Change the color of either FLP-15 or dopamine. They are difficult to distinguish in the figure.
11. Line 455: The authors have published a paper on the role of FLP-15 in locomotion previously. Hence, this sentence is not correct.

Reviewer #3

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Communications Biology initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4

(Remarks to the Author)
The manuscript entitled "Flp-15 functions through the GPCR NPR-3 to regulate local and global search behaviours in Caenorhabditis elegans" by Bhat et al. identifies flp-15 as a key regulator required for the behavioural switch between local and global foraging, particularly in modulating reversal frequency. The authors demonstrated that FLP-15 acts through the I2 neuron via the GPCR NPR-3, which functions in the dopaminergic neurons ADE and CEP. Overall, the manuscript is clearly written, well organised, and presents novel insights into the role of FLP-15 in coordinating foraging behaviour. The findings are likely to be of interest to the readership of Communications Biology. Below I outline several experiments and analyses that I think are important for establishing the authors' proposed mechanism as well as a few minor comments that should be addressed before publication.

Major comments

1. The authors justify their focus on flp-15 based on its reduction in reversal frequency during the local search phase compared to wild-type animals, and its failure to decrease reversal frequency upon transitioning from local to global search. However, several other mutants also appear to display a similar phenotype, albeit to a lesser extent. Upon closer examination of figure 1, the reversal frequency of flp-15 mutants seems to remain relatively constant between the local and global search phases. Other mutants, such as flp-6 and flp-3, also appear to exhibit similar patterns. It would therefore be helpful for the authors to clarify why these mutants were not further investigated. These strains may, like flp-15, reflect a lack of adaptive response to food deprivation and could provide additional mechanistic insights.

Furthermore, since flp-15 mutants appear unresponsive to food cues and speed remained constant throughout (Figure S2B), it is possible that they exhibit a general defect in locomotion control rather than a specific behavioural deficit related to food. To address this, the authors could include an assay demonstrating that locomotion control remains intact, for example, by showing that flp-15 mutants can increase their speed in response to a non-food stimulus such as blue light.

2. Regarding flp-15 expression levels. Did the authors measure flp-15 expression levels under fed conditions (or time 0, before the onset of local search)? This could further strengthen the conclusions by providing a baseline reference point.

3. Regarding flp-15 rescue experiment. I assume that the construct is maintained as an extrachromosomal array, where expression levels may exceed endogenous levels. If flp-15 expression increases during food deprivation, one might expect that overexpression would drive worms into a constant state of global exploration. However, since the extrachromosomal expression rescues the flp-15 phenotype, this argues against expression level alone being responsible. If feasible, measuring flp-15 expression in the rescue strain (maybe flp-15-gfp) could provide additional support for the conclusion reached.

4. Pharyngeal pumping experiment. Measurements were performed after acclimating the worms under blue light for one minute. Could the authors clarify the rationale for this choice? It is established that blue light inhibits pharyngeal pumping through activation of the I2 neurons via GUR-3, and through LITE-1 in other neurons. Therefore, exposure to blue light could artificially suppress pharyngeal activity. I suggest repeating the experiment under red filtered illumination to eliminate possible interference from LITE-1 and GUR-3.

5. If the authors wish to support the idea that dopamine levels are involved in flp-15/npr-3-dependent modulation of foraging behaviour, additional evidence such as measurements of dopamine levels in ADE and CEP neurons would help substantiate this claim.

6. Starting at line 412, the statement "These data suggest that a decrease in dopamine levels may be required to regulate

the frequency of reversals during local and global search behaviours" is somewhat confusing. Since cat-2 mutants are already in a chronically reduced dopamine state and the switch still occurs, it is unclear whether this effect can be solely attributed to dopamine levels. Consider revising this statement for clarity.

7. Starting on line 426, the authors discuss the cat-2 and flp-15 mutant results in terms of roaming dwelling states. These should also be accompanied by differences in speed. Are the speeds of these mutants also consistent with roaming and dwelling? Given the role of dopamine in worms' slowing response on food, is there a defect in speed on food in either mutant?

Minor comments

1. "G-protein couple receptor" should be "G protein-coupled receptor"

2. Figure 1: To aid comparison, I suggest combining the local and global search data into a single graph, allowing direct visual comparison between the two behavioural states.

3. Figure 2: The colour choice for box plots isn't consistent, and this can be a bit confusing. I would recommend using consistent colours for WT vs. mutant or not using colour at all.

Reviewer #5

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Communications Biology initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have addressed most of our concerns satisfactorily. We only have a few minor comments:
- Figure 2B shows MSD, but the units are non-standard (mm^2/min instead of just mm^2). The authors cite another paper to understand how this is computed, but given that it's a non-standard metric, we think that they should explain in Methods how exactly this is computed, including what time points were chosen to compute the MSD, and how it is normalized to yield mm^2/min.
- For example in line 273, the authors write "reversal frequencieS". We were a bit confused by the use of plural (we thought they might refer to several types of reversals?). If this is just the frequency of reversals, we think that singular would be clearer.
- On line 471, the authors still use the terms "local" and "global" search in a way we find a bit confusing: cat-2 mutants show more reversals at 25-30 minutes than the WT during 0-5 min, so we're not sure it's appropriate to use these terms. If the authors have a good reason to keep it here we're ok with that, but we would recommend using the time windows instead.
- In line 460, the authors say that cat-2 mutants show increased reversal frequency and enhanced exploratory behavior. In the subsequent discussion they equate higher reversal rate with higher exploration, but shouldn't higher reversal rate lead to less exploration? If so, aren't these results contradictory with the effect of dopamine on dwelling?

Reviewer #3

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Communications Biology initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4

(Remarks to the Author)
The authors have addressed all our comments. Just one typo to fix:
Line 560: provide and indirect -> provide an indirect

Reviewer #5

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Communications

Biology initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

We sincerely thank the Editor and all Reviewers for their careful evaluation of our manuscript and for the constructive comments. These comments provided a positive assessment of the work and we have revised the manuscript carefully to improve clarity, rigor, and interpretation. Below, we address each comment point-by-point. All changes are highlighted in blue in the revised manuscript.

Reviewer #1

We find this to be a solid and interesting paper. It claims that the transition from local to global search in C. elegans is regulated by FLP-15 through the I2 pharyngeal neuron via the NPR-3 receptor. These claims appear to be well supported by the data presented, though we would like to note that we are experts in C. elegans behavior, so our review focuses on the behavioral experiments and on the logic when interpreting the results from the different mutants; we are not specialists in neurobiology or genetics. From our point of view, the paper is adequate for publication in this journal, and we mainly offer minor suggestions that we hope may improve the paper's clarity.

We thank the Reviewers for their time and are happy that they found this to be a solid and interesting manuscript. We thank them for their useful suggestions and have tried to address all their points below.

Major Suggestions:

1. The main claim of the paper refers to the transition between local and global search, and the paper uses the frequency of a specific type of reversal (>2 head swings) as a proxy of the type of search. While this is a very reasonable proxy and all results are consistent with what we know about local/global search in C. elegans, the claim would be strengthened by a control showing that this proxy is indeed a good indicator of the type of search. To do this, the authors could show for example the Mean Squared Displacement in the two relevant periods (0-5 min and 25-30 min), and show that it correlates with the frequency of reversals. Including this analysis for both wild-type and mutant strains would further reinforce their argument.

Response: We agree that spatial metric strengthens the behavioural interpretation of reversal frequency as a proxy for search strategy. We have now performed Mean Squared Displacement (MSD) analysis during the 0-5 min and 25-30 min periods for wild-type and *flp-15.* This analysis demonstrates that reversal frequency correlate with spatial exploration across these periods, supporting the use of reversal frequency as a valid indicator of local versus global search behaviours. These data are now included in Figure 2B and discussed in lines 290-298, 505-510 and 809-817.

2. The terms "local" and "global" search are used throughout the manuscript, including the Methods and Results sections, in a way that we fear may be misleading. In some

places the authors seem to use "local search" as a synonym of what happens in the 0-5 min period and "global search" as a synonym of what happens in the 25-30 min period, even when the worm's behavior does not actually change (for example, lines 272-275). The terms "local search" and "global search" should be reserved for the cases where C. elegans actually shows these movement patterns, so we suggest reporting results using objective time intervals (e.g., 0–5 min and 20–25 min) and discussing local/global search in the Discussion, rather than implying direct measurement of the phenomenon in the Methods or Results.

Response: We appreciate this important point and agree with it. We have revised the manuscript and have now used time windows where the worms fail to show the behavioural transition and reserve the terms "local search" and "global search" appropriately. Examples in lines 373-375 and 488-490.

3. Line 273: The authors state that the flp-15 mutant failed to reduce reversal frequency when transitioning from local to global search. However, according to Figure 2A, the issue seems not to be a failure to reduce, but rather a failure to exhibit the high initial reversal frequency observed in the wild type. The mutants maintain a constant low reversal frequency throughout the assay, suggesting that the local-to-global search transition might be absent in these animals.

Response: We agree and thank the reviewer for this clarification. We have revised the text to state that *flp-15* mutants fail to produce a high-reversal exploratory state during the local search, resulting in an absence of a transition rather than a failure to reduce reversals. This interpretation is now consistently applied throughout the manuscript.

4. Lines 340-341: Similarly, the authors state that the npr-3 mutants failed to exhibit a high reversal frequency during the local search phase, which raises the question of how comparable this behavior is to what is typically defined as local search. In addition, the authors report that these mutants did not show a temporal decrease in reversal frequency during the "transition from local to global search," which could be confusing given that the Introduction describes this transition as being primarily driven by a decrease in reversal frequency.

Response: We agree and have revised the manuscript to state that *npr-3* mutants do not exhibit canonical local search behaviour, as defined by increased reversal frequency. We no longer describe their behaviour as a "transition from local to global search". Instead, we now state that *npr-3* mutants are unable to generate the elevated reversal frequency characteristic of the early off-food exploratory phase, resulting in the absence of a detectable temporal modulation in reversal frequency (lines 373-375).

Minor suggestions:

Reversal quantification: The authors quantify reversals using events longer than two head swings; however, it is unclear how representative these are of the total number of reversals executed by the animals. How was this threshold chosen? How representative are two-head-swing reversals, relative to all reversal events?

Response: We thank the reviewer for raising this important point. The threshold of reversals longer than two head swings was chosen based on prior behavioural analyses demonstrating that longer reversals are selectively enriched during off-food exploration and are closely associated with reorientation behaviours characteristic of local search. In particular, Gray et al., 2005 showed that reversals with longer temporal duration, typically comprising three or more head swings increase following removal from food, whereas short reversals are common on food and decline rapidly after food removal. Consistent with these findings, Gray et al. reported that immediately after removal from food, the frequency of short reversals decreased, while long reversals (≥3 head swings) increased 10-20 fold and remained elevated during off-food exploration. We therefore focused our analyses on reversals exceeding two head swings, as these events capture the reorientation events associated with off-food search.

We have now clarified this rationale in the Methods section and stated that while shorter reversals do occur, reversals exceeding two head swings represent a behaviourally relevant and well-established subset of reversal events to distinguishing search strategies (lines 187-193).

Mapping figures to experiments: It would help readers if a clearer connection between figures and the corresponding experiments in the Methods section were provided, for example by specifying whether it was a single tracking experiment or a multi animal tracking experiment. Currently, it is difficult to determine which experimental data corresponds to each figure (for example, in Figure 2 it is not clear if data come from single animal experiments or not).

Response: We agree that the link between figures and the corresponding experimental paradigms was not sufficiently clear. We have now revised the Methods to specify, for each experiment, whether data were obtained from single-animal tracking or multi-animal tracking assay (line 167).

Figure color scheme: Consider using a consistent color scheme across all figures. The current use of colors is sometimes confusing, as the same color may represent different aspects in different figures. For example, in Figure 2A gray/blue represent

different time intervals but in Figure 2C they represent different strains. It would greatly improve readability to have a more consistent color scheme.

Response: We agree that inconsistent colour usage reduced figure clarity. We have now revised figures to use a consistent colour scheme across the manuscript, such that colours represent the same experimental variables throughout (e.g., genotype or condition).

Figure scale: Wherever possible, especially when displaying results for the 0–5 min and 20–25 min intervals in parallel plots, please use identical axis scales to facilitate visual comparison (For example, Figure 2C, D).

Response: We thank the reviewer for this helpful suggestion. In several cases, reversal frequencies during the 0-5 min and 20-25 min intervals differ substantially, making it difficult to use identical axis scales. However, we have been careful to maintain consistent axis scaling within each condition across figures.

Statistical significance: It would be helpful to clarify how statistical significance is represented in the figures. For example, in Figure 4 it is unclear what the asterisks on top of individual boxes mean. Is it significance with respect to WT? If so, this should be explicitly explained in the figure caption.

Response: We agree and then reviewers for this comment. We have now revised the methods to explicitly state that comparisons on top of each bar are with respect to WT (lines 257-259), other asterisks are indicated by brackets in the graphs.

<u>More Specific comments / suggestions for the Authors</u>

Abstract

•*Line 34: The phrase "in C. elegans" seems unnecessary given that in previous line authors already specified "in C. elegans".*

Thank you. We have revised the line and omitted the redundant use of "*C. elegans*".

•*Line 35: Please clarify what is meant by "cumulative reorientations" and how these can decrease if they are cumulative.*

We thank the reviewer for pointing out this ambiguity. Our use of the term "cumulative reorientations" may have been misleading. We refer to the overall frequency of reorientation behaviours, including reversals, upsilon turns, and omega turns, which are prominently executed immediately after removal from food and decline over time during off-food exploration (Lopez-Cruz et al., 2019). We have revised the text to replace "cumulative reorientations" with "reorientation frequency" (lines 34-35).

Introduction

•Line 80: Please either clarify what is meant by the "stereotypic locomotion of C. elegans" or consider omitting the term stereotypic.

Thank you. We have omitted the term stereotypic.

•Line 90: This sentence repeats information already mentioned in line 78.

Thank you. We agree this repeats the already provided information. We have removed this line in the revised manuscript (line 89).

•Lines 96-99: A citation is needed to support this claim. These vertebrate-specific peptides do not exist in C. elegans, which may confuse readers. In this species, roaming and dwelling behaviors are mainly modulated by serotonergic and dopaminergic signaling.

We thank the reviewer for this comment and agree that the original wording could be misleading, as the peptides referenced are vertebrate-specific and not present in *C. elegans*. We have revised this section, and have added the appropriate citations to support the statements (lines 95-98).

•Lines 101–103: This passage is hard to follow; please rephrase for clarity.

We thank the reviewer for this suggestion. We have revised the passage in lines 100–101 to improve clarity and readability

Methods

•Line 132: Specify the age of the worms or clearly define what is meant by "young adult".

We thank the reviewer for this comment. We have now clearly defined "young adult" in the Methods section by specifying the developmental stage as 48 hours post hatching (lines 132-133).

•Line 165: Consider omitting one of the "single" as there are two of them very close to each other.

We thank the reviewer for noting this redundancy. We have revised the sentence in to remove the repeated use of the word "single".

•Line 170: Consider tone-down the sentence as it may not be possible to eliminate all bacteria within a 2-minute crawling time window.

Thank you. We have revised the sentence in line 171 to use more cautious language.

•Line 171: The rationale for omitting cholesterol from the NGM plates should be clarified, since cholesterol can influence C. elegans physiology and behavior. Please explain why cholesterol-free media were used in experimental plates.

We thank the reviewer for raising this important point. Cholesterol-free NGM plates were used to eliminate an external source of metabolic sterols, thereby ensuring a consistent off-food condition during foraging assays. This approach minimizes variability arising from residual dietary components and helps maintain a uniform metabolic state across experimental animals during exploration. We have now clarified this rationale in the Methods section (lines 172-173).

•Line 175: C. elegans should be italicized.

Thank you. We have italicised *C. elegans* in the revision.

•Line 178: The phrase "under similar conditions" is ambiguous. Please clarify what aspects were kept constant between the initial and final recordings (e.g., same plate, temperature, illumination, and handling). Was the plate moved from the recorder between the first and the same period, or was it recorded continuously? This will ensure reproducibility and avoid confusion about potential manipulations during the 25-minute exploratory period.

We thank the reviewer for highlighting this ambiguity. We have revised the Methods section to clarify that the same assay plate was used throughout the experiment and that the plate was temporarily removed from the recording setup and placed back for the final recording period. Both recordings were performed under identical conditions, including temperature, illumination, and handling. These details have now been explicitly stated to ensure reproducibility and to avoid confusion regarding potential experimental manipulations during the intervening exploratory period (lines 181-182).

•Line 179: Possible incorrect reference to Figure S2A.

We thank the reviewer for pointing this out and apologize for the incorrect reference. The figure citation in line 182 has been corrected to Figure S1A.

•Line 184: Consider citing Chiba, C. M., & Rankin, C. H. (1990) to clarify what is meant by a "spontaneous reversal".

We thank the reviewer for this helpful suggestion. We have now cited Chiba and Rankin (1990) in line 186.

•Line 191: As mentioned above, the rationale for omitting cholesterol from the NGM plates and replacing agar with agarose should be clarified, as both modifications could potentially influence worm physiology and behavior, including pumping rate.

We thank the reviewer for this comment. As noted above, we have clarified the rationale for using cholesterol-free NGM plates in the Methods section. In addition, agarose was used in place of agar to provide improved optical clarity for high-resolution imaging pharyngeal pumping, a practice commonly employed in *C. elegans*

imaging experiments (line 203). To ensure that this substitution did not confound behavioural measurements, all experimental conditions including controls were performed on identical agarose-based media.

•*Line 193: As mentioned above, the authors should clarify what "young adults" refers to.*

We thank the reviewer for this comment. As noted above, we have already clarified and revised the definition of "young adults" in the Methods section by specifying the developmental stage and age of the animals used (lines 132-133).

•*Line 195: How long did the starvation period last? In case it is 1 minute consider a more appropriate term other than starvation, which is often reserved for longer periods.*

We thank the reviewer for this comment and agree that the term "starvation" may be misleading for short food-deprivation periods. We have now revised the text (line 206).

Results

•*Line 255: consider in text citation of Figure S1A.*

We could not understand what this meant, but are happy to make Figure S1A as in text citation.

•*Lines 274-276: This passage is hard to follow as local and global search are not conditions.*

We thank the reviewer for this comment. We have revised the passage that was in lines 274–276 to improve clarity and logical flow (lines 315-316).

Discussion

•*Lines 457-461: The authors present as two distinct results the observations that flp-15 mutants do not exhibit a high reversal frequency at the beginning of the experiment and that their reversal rate does not decrease over time. However, these two findings appear to describe the same phenomenon, since a reduction in an already low rate cannot be meaningfully detected. We suggest rephrasing this section (and any related statements elsewhere) to avoid presenting these as independent results.*

We agree with the reviewer that these observations reflect the same underlying phenomenon. We have revised the text (and related statements elsewhere in the manuscript) to avoid presenting these findings as independent results, and instead describe them as a single behavioural deficit (lines 488-490).

•*Lines 524-525: Please note that dwelling and roaming are behavioral states typically defined for on-food conditions and should not be conflated with off-food foraging or food-seeking patterns.*

We thank the reviewer for this important clarification. We have revised the text to avoid conflating on-food roaming and dwelling states with off-food foraging or food-seeking behaviours, and now describe these behaviours using appropriate, context-specific terminology (line 563-565 and Figure 6 legend lines 877-878).

Figures

•*Figure 1: It is a bit confusing that violin plots go below zero, given that a negative number of reversals cannot occur.*

We thank the reviewer for pointing this out. The apparent extension of the violin plots below zero resulted from kernel density smoothing at the distribution extremes rather than representing negative values (lines 804-805).

•*Figure 2: i) The authors refer to a transition from local to global search even though flp-15 does not show a decay in reversal frequency. This could be misleading, as such a transition may not occur in this strain. ii) Including, an explicit spatial coverage metric for the first 5 minutes and the last 25 minutes would substantially strengthen the argument. iii) Caption (B): The statement that there is "no difference" between on food and off food conditions seems inconsistent with the data, which show a visible difference.*

We thank the reviewer for these insightful comments and have revised the manuscript accordingly.

(i) We agree that describing a "transition from local to global search" in *flp-15* mutants is misleading. We have therefore revised the text and figure legends to avoid using this terminology for *flp-15* mutants and instead describe their behaviour in terms of the absence of an early elevated reversal phase.

(ii) To strengthen the behavioural interpretation, we have now included an explicit spatial coverage metric (Mean Squared Displacement) for the early (0-5 min) and late (25-30 min) periods. These data are now presented in Figure 2B and support the relationship between reversal frequency and spatial exploration (lines 290-298, 809-817).

(iii) We agree that the statement in the original Figure 2B caption was not clear. The legend text has been revised to accurately reflect the observed difference between on-food and off-food conditions (lines 809-812).

•*Figure 3: i) The figure shows four boxplots, but the caption describes only three treatments (wild type, flp-15, and the transgene). Please clarify this discrepancy. ii) It*

is also unclear whether these experiments were performed individually or collectively; please specify.

We thank the reviewer for pointing out this discrepancy.

(i) The fourth boxplot corresponds to the previously described rescue line (P*flp-15*::flp-15), which was introduced and discussed in an earlier Results section. In the section accompanying this figure, we focused on the new neuron-specific rescue construct (*Pgur-3*::flp-15), which led to the abbreviated description in the caption. To avoid confusion, we have now revised the figure caption and main text to explicitly describe all four conditions shown (lines 831-832).

(ii) In addition, we have clarified in Methods and figure legend that these experiments were performed using single-animal tracking, with animals analysed individually rather than in groups (line 167).

•*Figure S2B: "speed" would be more accurate than "velocity" on the axis (since "velocity" usually refers to a vector).*

We thank the reviewer for this correction. The y-axis labels in Figure S2B have been revised from "velocity" to "speed".

•*Figure 4: i) The mutants appear to maintain similar reversal frequencies during the first 5 and the last 25 minutes of the experiments. Thus, describing this as a "transition from local to global search" may not be appropriate, since the data do not clearly support such a transition. ii) On line 782, after "background" there appears to be a typo as "in" should likely read "is". Consider removing the phrase "in these mutants," as it is redundant.*

(i) We agree with the reviewer that describing a "transition from local to global search" is not appropriate for these mutants, as their reversal frequencies remain similar across the early and late time windows. We have revised the text and figure legends associated with Figure 4 to avoid this terminology and instead describe the behaviour in terms of the absence of temporal modulation in reversal frequency.

(ii) We thank the reviewer for identifying the typographical error. The text in line 851 has been corrected such that "in" now reads "is", and the redundant phrase "in these mutants" has been removed.

•*Figure 6: Please clarify why the term dwelling is used here. Typically, dwelling and roaming refer to on-food behaviors and are defined based on locomotion speed rather than reorientations.*

We thank the reviewer for this important clarification. We agree that the terms dwelling and roaming are classically defined for on-food conditions and are typically distinguished based on locomotion speed rather than reorientation frequency. To avoid confusion, we have revised the text and figure legend associated with Figure 6 to remove the term "dwelling".

Reviewer #2

Animals use a variety of sensory cues to explore their environment and direct them towards food. C. elegans undergoes a stereotyped behavior in the presence and absence of food. In particular, in the absence of food, animals progress from dwelling in its local environment to exploring a larger area, a behavior called foraging, to find food. Previous work from this group and others have shown that FLP-15 peptides, which are released from the proprioceptive pharyngeal I2 neurons, signal through the NPR-3 receptor in dopaminergic neurons to affect locomotory behavior. flp-15 mutants have an increased amplitude and increased number of body bends during reversal compared to wild-type animals. However, it was unclear from these authors' previous paper (Bhat et al., 2025) whether what was scored as increased reversal length actually corresponded to an actual increase in the length of the reversal (as opposed to the number of head bends).

Response: We thank the reviewer for raising this point. In our previous paper (Bhat et al., 2025) Reversal length was quantified by measuring the number of head swings executed during a single reversal event, with longer reversals defined by an increased number of consecutive head bends before forward locomotion resumed. This approach is commonly used to distinguish short and long reversals and to assess the extent of reorientation during exploratory behaviour (Gray et al., 2005; Bhardwaj et al., 2011).

In this paper, the authors examine a switch from searching locally to globally for food during food deprivation. The authors look at the number of reversals, as opposed to number of body bends/reversals that was counted in their previous paper, made during searching. flp-15 mutants have a decreased reversal rate compared to wild-type animals in the absence of food. As on food, FLP-15 signals through the NPR-3 receptor expressed in dopaminergic neurons for this behavior. However, the authors did not indicate whether the number of body bends/reversal or distance traveled were affected. In addition, the number of reversals in flp-15 mutants do not appear to change over time without food. If the animals were recorded during the entire time, did flp-15 mutants show any change of behavior from 0-30 minutes?

Response: We thank the reviewer for these insightful comments. To address whether spatial exploration and behavioural modulation change over time, we have now performed Mean Squared Displacement (MSD) analysis during the early (0-5 min) and late (25-30 min) periods for both wild-type and *flp-15* mutants. This analysis demonstrates that reversal frequency correlates with spatial exploration across these periods, supporting the use of reversal frequency as a valid indicator of search behaviour. Importantly, while wild-type animals show a clear increase in spatial coverage over time, *flp-15* mutants exhibit minimal change in MSD between the early and late periods, consistent with their stable reversal frequency across the assay.

These data are now included in Figure 2B and discussed in lines 290-298, 505-510 and 809-817.

A novel finding was that levels of flp-15 mRNA increase the longer an animal is under starvation conditions, leading the authors to propose that off food, the levels of flp-15 mRNA increase as the levels of dopamine decrease. Does this hypothesis extend to when animals are returned to food? Would flp-15 mRNA levels return to basal levels? In addition, how dopamine levels will be decreased in response to increased FLP-15 production and the mechanism for this compensation is unclear.

Response: We thank the reviewer for these thoughtful questions. We have now examined *flp-15* mRNA levels after returning animals to food following 30 minutes of food deprivation. Under these conditions, *flp-15* mRNA levels decrease toward baseline, indicating that the starvation-induced upregulation of *flp-15* is dependent on food availability (Figure 2F and lines 321-325).

With respect to dopamine, while we have not directly measured dopamine levels, our genetic interaction analyses and dopamine supplementation assays provide indirect evidence that dopaminergic signalling is reduced in the absence of FLP-15. We agree that the precise mechanism by which increased FLP-15 production influences dopamine signalling remains unclear. We have therefore tempered our statements accordingly and now present this model as a working hypothesis, noting that ongoing and future studies in our laboratory aim to further dissect the underlying circuit mechanisms (lines 469-470 and 560-561).

Overall, the paper has some interesting new work, but the hypotheses need to be fleshed out better and more results included. For instance, I2 is a proprioceptive neuron that senses oxidative stress and light in the pharynx to inhibit feeding. Because pharyngeal rates are not affected in flp-15 mutants, can I2 neurons also be sensors for food volume (i.e., the neuron monitors how much the pharynx is distended with food) to regulate movement? What input is being received by I2 neurons to activate ADE/CEP neurons?

Response: We thank the reviewer for these thoughtful and insightful suggestions. We agree that the possibility that I2 neurons integrate additional sensory inputs such as pharyngeal distension or food volume to modulate locomotory behaviour is an intriguing hypothesis. While pharyngeal pumping rates were not altered in *flp-15* mutants, this does not preclude a role for I2 neurons in sensing other aspects of feeding or pharyngeal state.

Due to time constraints, we were unable to design and perform experiments specifically testing whether food volume or pharyngeal distension serves as an input to activate I2 neurons and influence ADE/CEP activity. We have therefore avoided speculative claims in the manuscript. However, ongoing work in our laboratory is

currently investigating potential changes in pharyngeal structure and physiology in *flp-15* mutants, which may provide further insight into how sensory information from the pharynx is integrated into this circuit.

Minor comments:

1. *The alleles of the strains examined in depth should be included in the Materials & Methods. Only one allele of flp-15 and npr-3 were tested. At least two alleles of each gene should be tested, especially as at least one other allele of npr-3 is available in the authors' lab.*

Response: We thank the reviewer for raising this point. For *flp-15*, only a single allele is available through the Caenorhabditis Genetics Center (CGC). This allele represents a complete knockout, with all known isoforms deleted, making it a strong loss-of-function for assessing FLP-15 function. For *npr-3*, no mutant allele was available through the CGC at the time of this study; therefore, we generated a CRISPR-Cas9-mediated *npr-3* knockout specifically for this work.

We agree that analysis of multiple independent alleles would further strengthen the conclusions. However, due to logistical constraints, we were unable to procure additional strains from alternative sources.

2. *Fig. 2B: Are the off food animals ones that were on food and then switched to off food? Or completely different animals?*

Response: We thank the reviewer for this clarification. In the current Figure 2E (previously part of Figure 2B) *C. elegans* were first tested for reversals on food and then transferred to an off food plate to plate to test for reversals at the 0-5 minutes time-point as described above (lines 198-200).

Supplementary Information, Table S1: all alleles should be italicized.

Response: We thank the reviewer for this correction. All allele names in Supplementary Table S1 have now been italicised.

3. *Lines 283, 307: Not supplementing flp-15, because it sounds like an additive to the petri dish. Transforming? Carrying the transgene?*

Response: We thank the reviewer for pointing out. We agree that the term "supplementing" was inappropriate in this context. We have revised the text in lines 283 and 307 to clearly state that *flp-15* was expressed via transgenic animals carrying the corresponding transgene (lines 310-312 and 340-342).

4. *For qPCR experiments indicate the housekeeping genes that were used for internal controls.*

Response: We thank the reviewer for this comment. For the qPCR experiments, *tba-1* was used as the housekeeping gene for normalization (lines 231-232).

5. *flp-15 expression in I2 has been previously reported and should be cited.*

Response: We thank the reviewer for this comment. We have now cited the relevant prior study reporting *flp-15* expression in the I2 neurons (line 336).

6. *Supplementary figure legends Line 51: Please indicate to what the arrow heads are pointing.*

Response: We thank the reviewer for this comment. And have indicated this in the figure legend (lines 56-58 in the supplementary file)

7. *Fig. 5 legend. Please indicate orientation of the animal (i.e., anterior is to the left? Ventral view?).*

Response: We have now revised the figure and indicated the direction of animal (lines 828-829 for Figure 3 and line 862 for Figure 5).

8. *Fig. 6C: Reverse the order of the bars. Minus dopamine should be before no dopamine.*

Response: We thank the reviewer for this suggestion. The order of the bars in Figure 6C has been reversed.

9. *Fig. 7: Change the color of either FLP-15 or dopamine. They are difficult to distinguish in the figure.*

   Response: We thank the reviewer for this suggestion. The colour scheme in Figure 7 has been revised.

10. Line 455: The authors have published a paper on the role of FLP-15 in locomotion previously. Hence, this sentence is not correct.

Response: We apologize for this oversight and thank the reviewer for pointing it out. The statement in line 455 has been corrected to mention our previously published work on the role of FLP-15 in locomotion (lines 485-486).

Reviewer #4

The manuscript entitled "Flp-15 functions through the GPCR NPR-3 to regulate local and global search behaviours in Caenorhabditis elegans" by Bhat et al. identifies flp-15 as a key regulator required for the behavioural switch between local and global foraging, particularly in modulating reversal frequency. The authors demonstrated that FLP-15 acts through the I2 neuron via the GPCR NPR-3, which functions in the dopaminergic neurons ADE and CEP. Overall, the manuscript is clearly written, well

organised, and presents novel insights into the role of FLP-15 in coordinating foraging behaviour. The findings are likely to be of interest to the readership of Communications Biology. Below I outline several experiments and analyses that I think are important for establishing the authors' proposed mechanism as well as a few minor comments that should be addressed before publication.

Response: We thank the reviewer for the positive and encouraging assessment of our manuscript. We are pleased that the reviewer finds the study clearly written, well organised, and of interest to the readership of *Communications Biology*. We have carefully considered the suggested experiments and analyses and have revised the manuscript accordingly to strengthen the mechanistic interpretation, as detailed in our point-by-point responses below.

Major comments

1. The authors justify their focus on flp-15 based on its reduction in reversal frequency during the local search phase compared to wild-type animals, and its failure to decrease reversal frequency upon transitioning from local to global search. However, several other mutants also appear to display a similar phenotype, albeit to a lesser extent. Upon closer examination of figure 1, the reversal frequency of flp-15 mutants seems to remain relatively constant between the local and global search phases. Other mutants, such as flp-6 and flp-3, also appear to exhibit similar patterns. It would therefore be helpful for the authors to clarify why these mutants were not further investigated. These strains may, like flp-15, reflect a lack of adaptive response to food deprivation and could provide additional mechanistic insights.

Furthermore, since flp-15 mutants appear unresponsive to food cues and speed remained constant throughout (Figure S2B), it is possible that they exhibit a general defect in locomotion control rather than a specific behavioural deficit related to food. To address this, the authors could include an assay demonstrating that locomotion control remains intact, for example, by showing that flp-15 mutants can increase their speed in response to a non-food stimulus such as blue light.

Response: We thank the reviewer for this thoughtful comment. In our initial screen, multiple neuropeptide mutants displayed alterations in reversal frequency consistent with the phenotype of interest. However, due to time and logistical constraints, it was not feasible to pursue detailed mechanistic analyses for more than one candidate for the present study. We therefore focused on *flp-15* because it exhibited the most robust and reproducible phenotype. We agree that other neuropeptides, including *flp-6* and *flp-3*, may also contribute to adaptive responses to food deprivation but feel they would be beyond the scope of this study.

To address the possibility that *flp-15* mutants may exhibit a general locomotor deficit, we performed an additional assay using a **non-food sensory stimulus (blue light)** to test whether locomotion control remains intact. The average speed of *flp-15* mutant

animals was comparable in presence and absence of blue light compared to WT animals in the respective blue light state. The increase in speed was also quantified and the results show that, although there is a smaller increase in the speed of *flp-15* mutants after blue light exposure, the difference in speed is not is not statistically significant (Figure S2C and D and lines 302-309 and 39-44 in the supplementary file).

2. Regarding flp-15 expression levels. Did the authors measure flp-15 expression levels under fed conditions (or time 0, before the onset of local search)? This could further strengthen the conclusions by providing a baseline reference point.

Response: We thank the reviewer for this suggestion. Yes, *flp-15* expression levels were measured under fed conditions, and the on-food (time 0) state was used as the baseline reference for all fold-change calculations shown in the manuscript. We have now clarified this explicitly in the Methods and Results sections to avoid ambiguity (lines 225-227).

3. Regarding flp-15 rescue experiment. I assume that the construct is maintained as an extrachromosomal array, where expression levels may exceed endogenous levels. If flp-15 expression increases during food deprivation, one might expect that overexpression would drive worms into a constant state of global exploration. However, since the extrachromosomal expression rescues the flp-15 phenotype, this argues against expression level alone being responsible. If feasible, measuring flp-15 expression in the rescue strain (maybe flp-15-gfp) could provide additional support for the conclusion reached.

Response: We thank the reviewer for this insightful comment. We agree that extrachromosomal arrays can result in elevated expression levels relative to endogenous conditions. Due to time constraints, we were unable to generate a FLP-15::GFP reporter line and perform quantitative expression analyses in the rescue background within the scope of this study. However, in both the present work and our previous study (Bhat et al., 2025), the rescue construct was injected at a low concentration of 35 ng/µl (line 144), and we did not observe phenotypes indicative of FLP-15 overexpression. Notably, the same rescue construct was previously shown to partially rescue the amplitude defect in *flp-15* mutants (Bhat et al., 2025), suggesting that its expression does not simply drive behavioural outcomes through excessive peptide levels.

4. Pharyngeal pumping experiment. Measurements were performed after acclimating the worms under blue light for one minute. Could the authors clarify the rationale for this choice? It is established that blue light inhibits pharyngeal pumping through activation of the I2 neurons via GUR-3, and through LITE-1 in other neurons.

Therefore, exposure to blue light could artificially suppress pharyngeal activity. I suggest repeating the experiment under red filtered illumination to eliminate possible interference from LITE-1 and GUR-3.

Response: We thank the Reviewer for their comment that allows us to clarify the measurement conditions. For these measurements we used PharaGlow, a previously published tool (https://elifesciences.org/articles/77252) and imaging protocol. Pharaglow imaging uses a much lower magnification and light intensity compared to usual single worm trackers. We had previously assessed the impact of excitation light on pumping behavior, and found no effect (Figure 2-figure supplement 5 from Bonnard and Liu et al., 2022). Likely, this is due to the specific light intensity and wavelength. The lite-1 receptor reacts most strongly to light in the blue/UV range. However, the receptor activity drops dramatically above 405 nm (https://pmc.ncbi.nlm.nih.gov/articles/PMC5388352/). Here, we use ~470nm LED excitation, which is far from the receptor response. However, as we detected a mild change in speed, we decided to acclimate the worms to blue light before the start of measurements. We have added a sentence to the methods referring to the prior data regarding the lack of pumping suppression by light under these conditions (lines 212-213).

5. If the authors wish to support the idea that dopamine levels are involved in flp-15/npr-3-dependent modulation of foraging behaviour, additional evidence such as measurements of dopamine levels in ADE and CEP neurons would help substantiate this claim.

Response: We thank the reviewer for this important suggestion. At present, we do not have direct measurements of dopamine levels in ADE and CEP neurons. We agree that such experiments would provide strong additional support for the proposed mechanism. Accordingly, we have toned down our claims regarding dopamine levels throughout the manuscript and now present dopaminergic involvement as a model supported by genetic and pharmacological interactions, rather than as a directly demonstrated change in dopamine concentration. We explicitly acknowledge this limitation in the Discussion and note that future studies from our laboratory will aim to directly measure dopaminergic activity within this circuit (lines 469-470 and 560-565).

6. Starting at line 412, the statement "These data suggest that a decrease in dopamine levels may be required to regulate the frequency of reversals during local and global search behaviours" is somewhat confusing. Since cat-2 mutants are already in a chronically reduced dopamine state and the switch still occurs, it is unclear whether this effect can be solely attributed to dopamine levels. Consider revising this statement for clarity.

Response: We thank the reviewer for this important clarification. We agree that the original statement overstated the role of dopamine levels alone. While *cat-2* mutants

do exhibit a temporal switch in behaviour, the magnitude of reversal frequency does not reach wild-type levels. As shown in Figure 6B, reversal frequency in *cat-2* mutants is significantly higher than in wild-type and *flp-15* mutants, indicating that although dopaminergic signalling contributes to regulating reversal frequency, it is not solely responsible for the observed behavioural modulation. We have therefore revised the statement to clarify that dopamine acts as a modulatory component, and that additional signalling pathways are likely to contribute to the regulation of reversal frequency during off-food exploration (lines 446-447).

7. Starting on line 426, the authors discuss the cat-2 and flp-15 mutant results in terms of roaming dwelling states. These should also be accompanied by differences in speed. Are the speeds of these mutants also consistent with roaming and dwelling? Given the role of dopamine in worms' slowing response on food, is there a defect in speed on food in either mutant?

Response: We thank the reviewer for this comment. We agree that roaming and dwelling are behavioural states classically defined under on-food conditions and are typically distinguished based on locomotion speed. To avoid conceptual confusion, we have revised the text remove the roaming/dwelling. Accordingly, we no longer interpret these results in terms of roaming or dwelling states, instead, we describe the observed phenotypes using off-food appropriate terminology.

Minor comments

1. "G-protein couple receptor" should be "G protein-coupled receptor"

Response: We thank the reviewer for noting this error. The term has been corrected to "G protein-coupled receptor" throughout the manuscript.

2. Figure 1: To aid comparison, I suggest combining the local and global search data into a single graph, allowing direct visual comparison between the two behavioural states.

Response: We thank the reviewer for this helpful suggestion. We considered combining the local and global search data into a single graph, however, doing so would substantially overcrowd the figure and reduce readability, particularly given the number of genotypes and conditions shown. To preserve clarity while still enabling comparison, we have retained separate panels and ensured consistent labelling and formatting across them.

3. Figure 2: The colour choice for box plots isn't consistent, and this can be a bit confusing. I would recommend using consistent colours for WT vs. mutant or not using colour at all.

Response: We thank the reviewer for this suggestion. We have revised Figure 2 to use a consistent colour scheme, such that wild-type and mutant conditions are represented by the same colours across all panels.

Reviewer #1

The authors have addressed most of our concerns satisfactorily. We only have a few minor comments:

We thank the reviewer for the positive assessment of our revisions and for the additional helpful minor comments. We have addressed each point below.

Figure 2B shows MSD, but the units are non-standard (mm^2/min instead of just mm^2). The authors cite another paper to understand how this is computed, but given that it's a non-standard metric, we think that they should explain in Methods how exactly this is computed, including what time points were chosen to compute the MSD, and how it is normalized to yield mm^2/min.

Response: Lines 192-203, We thank the reviewer for this important suggestion. We have revised the Methods section to clearly describe the MSD calculation. Locomotion was recorded for 5 minutes at 8 frames per second. Centroid coordinates (x,y) were extracted for each frame and calibrated from pixels to millimetres. MSD was computed using a lag time of 30 seconds, and squared displacement was calculated between positions separated by consecutive non-overlapping 30-second intervals throughout the recording, yielding 10 displacement measurements per animal. These values were averaged to obtain a single MSD value per worm. MSD values were normalized to mm^2/min by dividing by the 30-second and then multiplying by 60.

For example in line 273, the authors write "reversal frequencieS". We were a bit confused by the use of plural (we thought they might refer to several types of reversals?). If this is just the frequency of reversals, we think that singular would be clearer.

Response: Line 282, Thank you for bringing this to our attention. We have now corrected it and use frequency in singular form.

On line 471, the authors still use the terms "local" and "global" search in a way we find a bit confusing: cat-2 mutants show more reversals at 25-30 minutes than the WT during 0-5 min, so we're not sure it's appropriate to use these terms. If the authors have a good reason to keep it here we're ok with that, but we would recommend using the time windows instead.

Response: Thank you for pointing this out. We were unable to locate the suggestion in line 471, however we could find the usage of the local and global term in line 451 which could be confusing, we have now changed and used time periods instead.

In line 460, the authors say that cat-2 mutants show increased reversal frequency and enhanced exploratory behavior. In the subsequent discussion they equate higher

reversal rate with higher exploration, but shouldn't higher reversal rate lead to less exploration? If so, aren't these results contradictory with the effect of dopamine on dwelling?

Response: Line 468- We thank the reviewer for pointing out this inconsistency. We agree that an increased reversal rate would be expected to reduce exploration. In the original sentence, we intended to refer specifically to enhanced local search behaviour during the 0-5 minute time window rather than increased exploration. We understand that this wording was misleading and could be interpreted as contradictory to the established role of dopamine in promoting dwelling behaviour. To avoid confusion, we have revised the text and removed this portion of the sentence in the revised manuscript. The sentence now reads "We observed that *cat-2* mutant *C. elegans* exhibited increased reversal frequency" instead of "We observed that *cat-2* mutant *C. elegans* exhibited increased reversal frequency and enhanced exploratory behaviour".

Reviewer #4

The authors have addressed all our comments. Just one typo to fix:

Line 560: provide and indirect -> provide an indirect

Response: We thank the reviewer for the positive assessment of our revisions and we have made the typographic change (line 567).

Review summary

We find this to be a solid and interesting paper. It claims that the transition from local to global search in *C. elegans* is regulated by FLP-15 through the I2 pharyngeal neuron via the NPR-3 receptor. These claims appear to be well supported by the data presented, though we would like to note that we are experts in *C. elegans* behavior, so our review focuses on the behavioral experiments and on the logic when interpreting the results from the different mutants; we are not specialists in neurobiology or genetics. From our point of view, the paper is adequate for publication in this journal, and we mainly offer minor suggestions that we hope may improve the paper's clarity.

MAJOR SUGGESTIONS

1. The main claim of the paper refers to the transition between local and global search, and the paper uses the frequency of a specific type of reversal (>2 head swings) as a proxy of the type of search. While this is a very reasonable proxy and all results are consistent with what we know about local/global search in *C. elegans*, the claim would be strengthened by a control showing that this proxy is indeed a good indicator of the type of search. To do this, the authors could show for example the Mean Squared Displacement in the two relevant periods (0-5 min and 25-30 min), and show that it correlates with the frequency of reversals. Including this analysis for both wild-type and mutant strains would further reinforce their argument.
2. The terms "local" and "global" search are used throughout the manuscript, including the Methods and Results sections, in a way that we fear may be misleading. In some places the authors seem to use "local search" as a synonym of what happens in the 0-5 min period and "global search" as a synonym of what happens in the 25-30 min period, even when the worm's behavior does not actually change (for example, lines 272-275). The terms "local search" and "global search" should be reserved for the cases where *C. elegans* actually shows these movement patterns, so we suggest reporting results using objective time intervals (e.g., 0–5 min and 20–25 min) and discussing local/global search in the Discussion, rather than implying direct measurement of the phenomenon in the Methods or Results.
3. **Line 273:** The authors state that the flp-15 mutant failed to reduce reversal frequency when transitioning from local to global search. However, according to Figure 2A, the issue seems not to be a failure to reduce, but rather a failure to exhibit the high initial reversal frequency observed in the wild type. The mutants maintain a constant low reversal frequency throughout the assay, suggesting that the local-to-global search transition might be absent in these animals.
4. **Lines 340-341:** Similarly, the authors state that the npr-3 mutants failed to exhibit a high reversal frequency during the local search phase, which raises the question of how comparable this behavior is to what is typically defined as local search. In addition, the authors report that these mutants did not show a temporal decrease in reversal frequency during the "transition from local to global search," which could be confusing given that the Introduction describes this transition as being primarily driven by a decrease in reversal frequency.

MINOR SUGGESTIONS

Reversal quantification:

The authors quantify reversals using events longer than two head swings; however, it is unclear how representative these are of the total number of reversals executed by the animals. How was this threshold chosen? How representative are two-head-swing reversals, relative to all reversal events?

Mapping figures to experiments:

It would help readers if a clearer connection between figures and the corresponding experiments in the Methods section were provided, for example by specifying whether it was a single tracking experiment or a multi animal tracking experiment. Currently, it is difficult to determine which experimental data corresponds to each figure (for example, in Figure 2 it is not clear if data come from single animal experiments or not).

Figure color scheme:

Consider using a consistent color scheme across all figures. The current use of colors is sometimes confusing, as the same color may represent different aspects in different figures. For example, in Figure 2A gray/blue represent different time intervals but in Figure 2C they represent different strains. It would greatly improve readability to have a more consistent color scheme.

Figure scale:

Wherever possible, especially when displaying results for the 0–5 min and 20–25 min intervals in parallel plots, please use identical axis scales to facilitate visual comparison (For example, Figure 2C,D).

Statistical significance:

It would be helpful to clarify how statistical significance is represented in the figures. For example, in Figure 4 it is unclear what the asterisks on top of individual boxes mean. Is it significance with respect to WT? If so, this should be explicitly explained in the figure caption.

More Specific comments / suggestions for the Authors

Abstract

- **Line 34:** The phrase "in *C. elegans*" seems unnecessary given that in previous line authors already specified "in *C. elegans*".
- **Line 35:** Please clarify what is meant by *"cumulative reorientations"* and how these can decrease if they are cumulative.

Introduction

- **Line 80:** Please either clarify what is meant by the "stereotypic locomotion of *C. elegans*" or consider omitting the term stereotypic.
- **Line 90:** This sentence repeats information already mentioned in line 78.
- **Lines 96-99:** A citation is needed to support this claim. These vertebrate-specific peptides do not exist in *C. elegans*, which may confuse readers. In this species, roaming and dwelling behaviors are mainly modulated by serotonergic and dopaminergic signaling.
- **Lines 101–103:** This passage is hard to follow; please rephrase for clarity.

Methods

- **Line 132:** Specify the age of the worms or clearly define what is meant by "young adult".
- **Line 165:** Consider omitting one of the "single" as there are two of them very close to each other.
- **Line 170:** Consider tone-down the sentence as it may not be possible to eliminate all bacteria within a 2-minute crawling time window.

- **Line 171:** The rationale for omitting cholesterol from the NGM plates should be clarified, since cholesterol can influence *C. elegans* physiology and behavior. Please explain why cholesterol-free media were used in experimental plates.
- **Line 175:** *C. elegans* should be italicized.
- **Line 178:** The phrase "under similar conditions" is ambiguous. Please clarify what aspects were kept constant between the initial and final recordings (e.g., same plate, temperature, illumination, and handling). Was the plate moved from the recorder between the first and the same period, or was it recorded continuously? This will ensure reproducibility and avoid confusion about potential manipulations during the 25-minute exploratory period.
- **Line 179:** Possible incorrect reference to Figure S2A.
- **Line 184:** Consider citing Chiba, C. M., & Rankin, C. H. (1990) to clarify what is meant by a "spontaneous reversal".
- **Line 191:** As mentioned above, the rationale for omitting cholesterol from the NGM plates and replacing agar with agarose should be clarified, as both modifications could potentially influence worm physiology and behavior, including pumping rate.
- **Line 193:** As mentioned above, the authors should clarify what "young adults" refers to.
- **Line 195:** How long did the starvation period last? In case it is 1 minute consider a more appropriate term other than starvation, which is often reserved for longer periods.

Results

- **Line 255:** consider in text citation of Figure S1A.
- **Lines 274-276:** This passage is hard to follow as local and global search are not conditions.

Discussion

- **Lines 457-461:** The authors present as two distinct results the observations that *flp-15* mutants do not exhibit a high reversal frequency at the beginning of the experiment and that their reversal rate does not decrease over time. However, these two findings appear to describe the same phenomenon, since a reduction in an already low rate cannot be meaningfully detected. We suggest rephrasing this section (and any related statements elsewhere) to avoid presenting these as independent results.
- **Lines 524-525:** Please note that *dwelling* and *roaming* are behavioral states typically defined for on-food conditions and should not be conflated with off-food foraging or food-seeking patterns.

Figures

- **Figure 1:** It is a bit confusing that violin plots go below zero, given that a negative number of reversals cannot occur.
- **Figure 2:** i) The authors refer to a transition from local to global search even though *flp-15* does not show a decay in reversal frequency. This could be misleading, as such a transition may not occur in this strain. ii) Including, an explicit spatial coverage metric for the first 5 minutes and the last 25 minutes would substantially strengthen the argument. iii) Caption (B): The statement that there is "no difference" between *on food* and *off food* conditions seems inconsistent with the data, which show a visible difference.

- **Figure 3:** i) The figure shows four boxplots, but the caption describes only three treatments (*wild type*, *flp-15*, and the transgene). Please clarify this discrepancy. ii) It is also unclear whether these experiments were performed individually or collectively; please specify.
- **Figure S2B:** "speed" would be more accurate than "velocity" on the axis (since "velocity" usually refers to a vector).
- **Figure 4:** i) The mutants appear to maintain similar reversal frequencies during the first 5 and the last 25 minutes of the experiments. Thus, describing this as a "transition from local to global search" may not be appropriate, since the data do not clearly support such a transition. ii) On line 782, after "background" there appears to be a typo as "in" should likely read "is". Consider removing the phrase "in these mutants," as it is redundant.
- **Figure 6:** Please clarify why the term *dwelling* is used here. Typically, *dwelling* and *roaming* refer to on-food behaviors and are defined based on locomotion speed rather than reorientations.