The salinity preference of *Caenorhabditis elegans*

Tristan Cornelis

Department of Biology, Vrije Universiteit Brussel, Brussels, Belgium

Abstract

In this study, we tested the behaviour of *Caenorhabditis elegans* by assaying their salt preference on salt gradients across square plates. We confirmed the literature expectations that *C. elegans* prefers the salinity they were grown on but only when not stressed (e.g., not starved). The latter expectation was only partially confirmed because the worms that starved in high salinity only showed a slight aversion to high-salt, while those which were starved under normal salinity showed no difference in behaviour compared to those that were not starved. Next, we expected males to show a greater preference for high salinity than hermaphrodites, and strains to differ from each other in salt preference, but neither of these assumptions were met. Juveniles were found to be more attracted to salt than adults, which is not reported in the literature, but more research is needed for this to be conclusive. Lastly, the F_1 of a N2 x MY16 cross showed a lower preference for salt than the parental generation.

Keywords

C. elegans, nematodes, salinity, salt tolerance, salt preference, behaviour

Introduction

Since 1974, when Sydney Brenner started experimenting with the molecular and developmental biology of *Caenorhabditis elegans*, these organisms have been used exponentially in a wide array of studies, ranging from mutation characterisation, host – pathogen coevolution, mating system evolution and life-history theory (Gray & Cutter, 2014). *C. elegans* has a short lifespan and generation time, making it very useful for studying the effects of new drugs that are being developed to combat human diseases. It also was the first animal to have its whole genome sequenced.

C. elegans has more recently been used for experimental evolution studies, such as: adaptation to novel environments, directional selection on male frequencies under androdioecy, optimal outcrossing rates under androdioecy, the existence of initial inbreeding depression, the strength of directional selection on male competitive performance, etc. (Teotonio *et al.,* 2012). However, while differences between males and hermaphrodites are characterised, for example, the individual male-specific neurons are mapped (Walsh *et al.*, 2020), the evolutionary consequences of having two sexes are understudied.

An interesting and simple way to test some of the behavioural aspects of the model organism is by using assays where their behaviour is analysed under stress-conditions. These stress conditions can be: pH-level, salinity, water hardness and temperature (Khanna *et al.*, 1997). However, some of these factors are easier to test than others. In the field of *C. elegans* research, salinity is the main factor used in most behavioural assays, due to it being fairly easy to set up and having determined boundaries. For these reasons, we chose to test for behaviour on a salt-gradient. The optimal salinity for *C. elegans* is around 0.2M. (Khanna *et al.*, 1997). By setting up a salt gradient, it is possible to study the preference of *C. elegans* to different salt concentrations by tracking their movement across the gradient to match their preference. In this study, existing salt gradient protocols to use in the lab were first adapted, aiming to eventually characterise salt preference across different strains, genders and conditioning to different salt concentrations.

Multiple methods to establish salt gradients on agar nematode growth medium (NGM) exist in the literature, one possibility is using a NaCl plug. It involves making two agar plates, one with a standard agar solution, the other with a certain NaCl

concentration. These concentrations can vary from test to test, ranging from low concentrations such as 0.05M (Sakai *et al.*, 2013) to 0.2M (Mah *et al.*, 2016). In the experiments using a NaCl plug specifically, the standard salinity used was 50mM (Hart, 2006), however, since it has been shown that 0.3M is the highest salinity *C. elegans* can tolerate (Mah *et al.*, 2016), using a gradient that has a higher concentration of NaCl than 50mM could have an increased negative effect on viability, so using concentrations lower than 30mM is preferable.

Another method to assay NaCl preference involves creating quadrants of different salinities on a plate. Here, the concentration of salt used most often is 25mM, which is half of the plug technique. In this setup, two plates are prepared with different salinities. After this, the agar is cut in 4 parts and 2 quadrants of each type of agar are placed in an alternating way on 1 plate (Peymen *et al.*, 2019). Gaps between the quadrants are filled with normal NGM medium.

Preference is scored in the same way for both types of assays. Although the salt plug has a gradient and the quadrants do not, boundaries can be chosen that split the plate in zones of high and low concentration for the salt plug, so worms can be counted for each treatment. In both the salt plug and quadrant experiments, the worms' movement is calculated by a chemotaxis index:

$$n(high) - n(low))/n(total).$$

Worms that are completely attracted to a higher salinity will have a chemotaxis index of 1, and those repelled by high salinity will have a chemotaxis index of –1.

A third method to make a salt assay is to tilt a square plate and fill it halfway with normal agar. After it solidifies in this inclined position, a second layer of agar with higher salinity is poured over it while keeping the plate level (Weinberg, 1959). This is scored by dividing the plate in areas of decreasing salinity.

Gradient plates can be used 24-48 h after being setup (Hart, 2006). It is best to test the behaviour of adult synchronised worms, i.e. worms that have the same age, to reduce behavioural variability. Adult hermaphrodites are either placed one by one in the middle of a gradient plate, and their movement is recorded after 20 minutes (Sakai *et al.*, 2013), or many worms can be transferred simultaneously through a liquid solution (Hart, 2006).

Previous work has shown that *C. elegans'* salt preference is plastic and dependent on the environment they were previously fed. (Hiroki *et al.*, 2021) A positive effect is only observed when the conditioning happened on a plate with food, if *C. elegans* is grown on a high-salt plate without food, they will actively avoid it when placed on the gradient. (Lim *et al.*, 2018)

In our experiment, both the plug and square gradient assays were used in preliminary scoring experiments to test their efficiency. The quadrant method was not attempted because it involved more complicated techniques to make it work and scoring was dichotomous, in contrast to the gradient plates.

After this trial, the square gradient assay was chosen since it is simpler to score. It is a square with equal concentrations and surface area along one axis, which is in stark contrast to the unpredictable pattern that is created by using a wedge-shaped plug on a round petri dish. The square assay also has less variability when being set up than the plugs, since the plug can vary in size, location, dryness, and so on, especially if it is made on a different day than the bottom petri dish. It is also easier to set up than the plug method because it only uses one plate. Lastly, the square method has the least chance of contamination. While two types of agar are used together in both assays, in the square one they are both poured on the same plate, so there is no gel cutting and moving across the plates, which are contamination risks. Contamination is not a big problem in the experiments, seeing that bacteria or fungi do not have time to grow and affect the worms by the time the assay finished, but any uncontrolled variable should be avoided to minimize interference as much as possible.

Next, male *C. elegans*' behaviour is different from that of hermaphrodites (Fagan & Portman, 2014). When placed on a small patch of food, hermaphrodites will stay in place and eat while males will explore more, this is due to males and hermaphrodites being attracted to different types of odorants. Males also move faster and with a higher amplitude, i.e. how much they bend while moving (Fagan & Portman, 2014). Lastly, when high salt concentrations are coupled with starving conditions, males will remain attracted to the salt if there are also hermaphrodites present, while hermaphrodites will always be repulsed by the higher concentration of salt if it is linked to a starvation experience (Fagan & Portman, 2014). Using this information, we can test if behaviour of male worms on the salt-plates is different from the hermaphrodites.

Our goal was to first confirm the literature predictions that worms grown on a certain salt concentration, given normal circumstances, will prefer this concentration when transferred to a gradient, this was done using worms grown on high salt conditions and normal conditions and testing their behaviour on a gradient assay. Secondly, we wanted to expand on the literature by assaying wild-collected worms to see if they differ in behaviour. Thirdly, we tested if the behaviour of males is different from those of hermaphrodites. Lastly, we looked at variables such as time on gradient, age, generation and synchronisation to study their effects on *C. elegans'* behaviour. By studying salt-tolerance in this manner, these results can form the basis for future experiments.

Results

Interaction of salt and starvation

To understand the interaction between growth in high salt conditions and starvation, the chemotaxis index of worms that were grown on high salt and normal medium and were either starved or not on both media were compared. To prevent bias, only data from adult *C. elegans* were used (five days and older), only synchronized worms (worms of the same age) were used and data from the F_1-cross was left out.

The non-starved worms grown in high salinity (Sa/nSt) had the highest chemotaxis index (0.708). The second highest mean (0.385) was for worms grown in high salinity but starved (Sa/St). This still shows a slight bias towards high salinity, but it is less positive than those that were not starved. The mean indexes of worms grown on low salinity were very close together, with that (-0.865) of the starved (nSa/St) being slightly lower than that (-0.688) of the non-starved (nSa/nSt). This shows a clear bias against high salinity for both categories ($F= 112.3$ on 3 and 13 DF, $p < 0.001$).

Presence of males

The chemotaxis indexes between synchronised worms with and without males, in low salt, non-starved conditions were statistically identical ($p= 0.817$). Similarly, there was no statistical difference in high salinity ($p= 0.2222$) ($F= 0.06186$ on 1 and 7 DF, p-value= 0.8107).

In F_1, the mean indexes when males were present were the same as when they were not present. This was the case in high and normal salinity.

There was no data of males in starved conditions or as juveniles.

F1 vs parental lines

Non-starved, synchronised adults grown in high salinity were more attracted to salt than F_1s of a MY16 x N2 cross (mean 0.885 vs 0.082). The chemotaxis indexes of P and F_1 were both low when grown on normal salinity, with a mean of -0.688 and -1 respectively ($F= 108.7$ on 3 and 13 DF, p-value< 0.001).

Juveniles vs adults

For juveniles, we only had data to compare them to adults under non-starvation and, normal salinity. Again, the F1-data was left out. Only adults of five days old were considered.

The estimated mean of the adults was -0.664, showing a clear preference for low salinity. The estimated mean of the juveniles was 0.55, which shows a preference for salt. These were all non-synced and the juveniles were together on the same gradient-plate with the adults and counted separately ($F= 52.15$ on 2 and 12 DF, $p < 0.001$).

Effect of time of observation

We observed the worms to calculate the indexes at 3 moments, after 5 minutes, 30 minutes and 24 hours. At 24 hours, we only measured twice, so these measurements were left out since there was not enough data to draw a conclusion from, but results were not similar to the bulk of the data.

On all other variables, there was no significant difference in indexes recorded at 5 minutes or at 30 minutes. The biggest difference was for normal salinity, non-starved conditions for adults from the parental line. Here, the difference in means was 0.2975 ($F= 1.245$ on 2 and 9 DF; $p>0.05$).

Difference between strains

The only situation where multiple strains (adults, P) could be compared against each other were in normal salinity, non-starved conditions. No difference was found (F-statistic= 0.7709 on 3 and 8 DF, $p > 0.05$).

Discussion

Interaction of salt and starvation

In this experiment, we expected worms grown in a certain condition, here high or normal salinity, to be attracted to similar conditions when placed on a gradient plate (Hiroki *et al.*, 2021). However, when worms experience stressful conditions, they will actively avoid the circumstances in which they were grown (Lim *et al.*, 2018). We therefore expected the worms that were starved in high salinity to show a preference for low salt (a low chemotaxis index) and those starved under normal salinity to be attracted to salt (a high chemotaxis index).

These expectations were met in both cases where the worms were not stressed. Worms grown in high and normal salinity were strongly attracted to those same conditions (Figure 1). When looking at the worms grown in high salinity that were starved (Sa/St), a lower attraction than the non-starved is seen, but it is still one that is positive (Figure 1). The worms grown at normal salinity and starved showed no difference in behaviour to those who were not starved (Figure 1; nSa/St vs nSA/nSt). It could be that the effect of starvation on worms' behaviour is smaller than that of the condition they are raised in, but then there should still be a difference for the non-salt, starved worms.

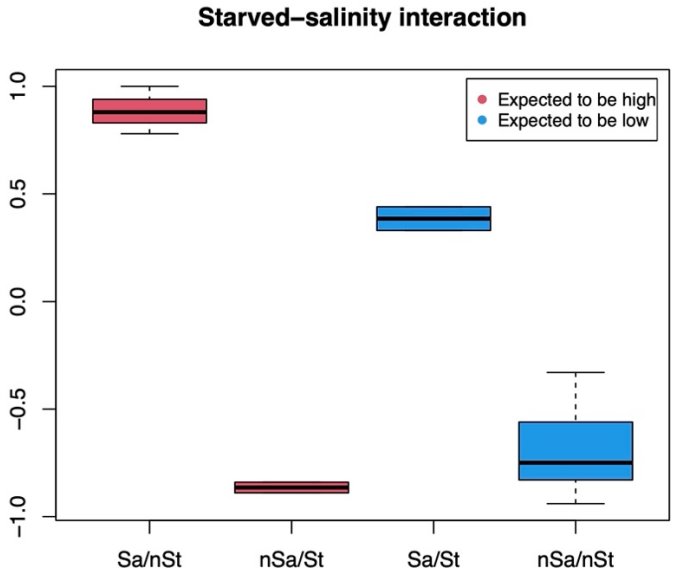

Figure 1: Boxplots of the interaction between starvation and salinity conditions for *C. elegans*

Presence of males

As previously mentioned, males are expected to move more and faster than hermaphrodites (Fagan & Portman, 2014). We therefore expected males to be attracted to salt more than hermaphrodites, since they would explore more. However, a difference in chemotaxis index between males and hermaphrodites was not found. One possibility for this discrepancy is low statistical power as, unfortunately, there were few males present in the assays performed. It is possible that another study with more males and more repeats of the assay would have found higher chemotaxis of males.

F1 vs parental lines

For this experiment, we had no expectation, but the F_1 generation of a MY16 x N2 cross showed a significantly lower attraction to salt. We only had 4 data points of the cross to compare. In future studies, this effect can be studied more extensively by doing more repeats but also by making crosses with more wild lines, and performing assays after more generations. If crossing shows a pattern of changing attraction to salt, it would form the basis of future analysis on the genetic basis of chemotaxis strength.

Juveniles vs adults

In standard non-starved, normal-salinity conditions, juvenile *C. elegans* showed a greater attraction to

salt than adults on the same plate. There is limited relevant data to this observation in the literature. One possibility is that it is the consequence of the fact that worms move slower with age (Michael P. Gardner, 2004) so it would be expected for juveniles to be more spread out on the gradient compared to the adults, but this does not explain why they show a preference for salt.

It is possible that there is an interaction between adults and juveniles when placed on a gradient simultaneously, so repeating this experiment with juveniles and adults separately is necessary to further explain this behaviour.

Another possibility is that the adult worms simply did not yet have time to reach their preferred salinity, and the juveniles seemingly liking salt more would be purely because of them being faster. To rule this out, more observations over an extended period of time could be performed.

The difference in salt aversion between juveniles and adults in different conditions (Starved, salt, F_1, etc…) could also be studied to fully understand the effect of age.

Difference between strains

In the data, of the 30 indexes belonging to P and adult worms, 18 were from N2's, the other 12 from 6 other strains. N2 is the standard strain used in scientific research, so we expected this to behave as in existing literature. Other strains were used supplementary, but no verifiable difference from N2 was found in this small data set. More assays comparing the strains with each other in more detail could find patterns not yet picked up by our data.

Conclusion

As expected, there was a positive correlation between the salinity *C. elegans* was raised in and the salinity they gravitate towards when placed on a gradient. Starving in high salinity lessened this effect slightly, while starving in low salinity did not influence their behaviour at all. For the N2 strain, salt-preference in males did not differ from that of hermaphrodites, but more and/or different assays may reveal changes in behaviour that could be used to study sexual adaptation. The preference of the worms was not affected by observation time (5 or 30 min post transfer to assay plate) suggesting they move very fast to conditions they prefer. A cross between males of N2 and L4's (virgins) of MY16 produced an F_1 that showed a lower attraction towards salt when raised on salt and normal-salt plates. Due to the limited data for this assay, a sampling error needs to be excluded by performing more assays. No difference between strains was found in salt preference. We did, however, find a higher preference for salt in juveniles compared to adults in several strains, but we could not rule out the result being due to faster movement of juveniles, and future assays should repeat the counting over a longer time.

Materials and methods

Strains used

12 strains, including the standard lab strain (N2) strain (Table 1) from the *Caenorhabditis* Natural Diversity Resource (CeNDR) (Crombie, 2024) were provided by the Liesbet Temmerman lab at KU Leuven. They were maintained at 20°C on nematode growth medium (NGM). The preliminary experiments were conducted on the N2 strain.

Table 1: strains legend

Strain	Location	Altitude	Landscape	Substrate
CB4856	Honolulu	60m	rural garden	rotting nut/ pod/ seed/ fruit
CX11314	Los Angeles	204m	urban garden	soil
DL238	Hawaii	653m	forest	vegetal/litter
ED3017	Edinbugh	85m	urban garden	compost
EG4725	Amares Portugal	101m	agricultural land	rotting nut/ pod/ seed/ fruit
JT11398	Seattle	124m	urban garden	compost
JU258	Fuccal	1041m	agricultural land	soil
JU775	Lisbon	66m	botanic garden	rotting nut/ pod/ seed/ fruit
LKC34	Mahajeby, Madagascar	686m	/	rotting nut/ pod/ seed/ fruit

MY16	Munster	61m	urban garden	compost
MY23	Munster	74m	urban garden	compost
N2	Bristol	9 m	urban garden	compost

Maintaining lines

Maintaining worm lines isn't limited to only one method. *C. elegans*' lifespan is usually around two weeks, so transferring worms from plate to plate once a week was sufficient for maintaining populations with multiple stages existing simultaneously. Alternatively, worms could be kept for several months in a diapause-like 'dauer' stage (Gray & Cutter, 2014). This stage is induced by starvation and causes worms to stop feeding and increase their lifespan almost indefinitely. Lines could also be frozen with glycerol, kept at –80° C indefinitely, and revived for future use (Hart, 2006).

Maintaining males and crossing lines

Males were maintained in a different way than the standard strains. Males reduce in frequency without selection, depending on the strain and genetic background, because males reach sexual maturity slower than hermaphrodites (Gray & Cutter, 2014). To maintain males, it was necessary to hand pick 8 males to a new plate, together with 2 L4 (pre-adult virgin) hermaphrodites. This way, the mature males were able to fertilise the hermaphrodites and the F_1 had a 50% male occurrence. Crossing between lines is an easy way to induce males from different genetic backgrounds and was done in the same way. F_1 then was 50% male and had 50% of the N2 DNA and 50% of the DNA of the other strain.

Synchronisation

Worm synchronisation allows to obtain large numbers of individuals of the same age, minimising behavioural variability. It was done by using bleach to dissolve adults and release eggs, which were washed and hatch within a small timeframe. Harsh bleach solution (1.25 ml 10M NaOH in 5 ml of NaCl (6-14% active chlorine (Merck)) was diluted 1:1 with distilled water immediately before use. Adults were moved to the side of a plate with food and 10 µl of diluted bleach solution was dropped on them. Old MGM plates were used to allow quick absorption of the bleach drop, because they were drier. The next day the emerged worms that had moved to food were placed onto a new plate.

Square gradient setup

60 mL of standard Agar-solution was poured on a square plate sitting at an incline. The angle was chosen so that the agar on the lower side of the plate touched the top, whilst the agar on the higher side barely covered the edge. The tilt was achieved by placing a 2 euro coin, two 20 cent coins and one 10 cent coin stacked on top of one another other under the edge of one side of the plate (Figure 2). This agar was allowed to dry for 1 hour, before taking away the coins and pouring 60mL of high NaCl concentration agar on top. After this, the salt gradient was slowly formed by diffusion and was ready to use after 24 hours .

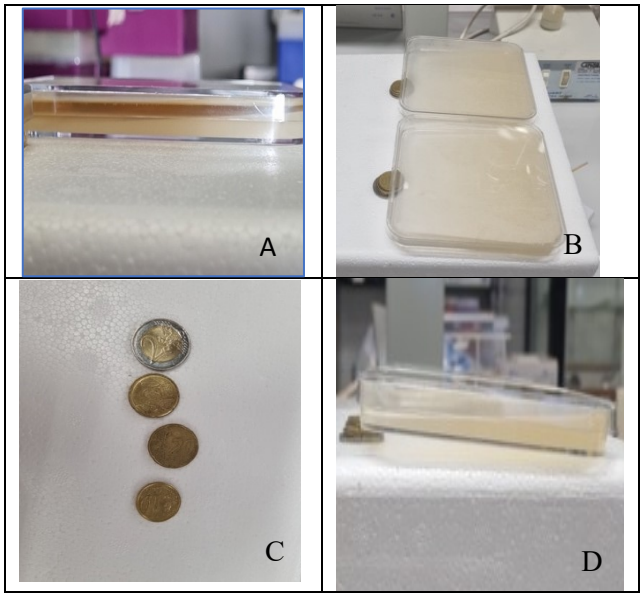

Figure 2; A: dried gradient. B: positioning while drying. C: coins used in setup. D: setup with one layer drying.

Transferring worms to gradient

To test the worms' behaviour, all worms from a synchronized plate were placed onto the square-gradient plate after 5 days of growing. This was done by washing the plate with M9 buffer and transferring the liquid to a 1.5 ml Eppendorf tube using a pipette. This Eppendorf tube was centrifuged for one minute at 3000 rpm, after which the supernatant was removed and more M9 was added. After removing most of the supernatant from the Eppendorf tube, a glass pipette was used to transfer the worms to the

gradient plate. The worms were placed in a horizontal line parallel to the salt gradient, on the middle of the gradient plate (area 3, Figure 2.). Because of the liquid, most worms could not move on the plate, so a paper tissue was dipped onto the plate to remove the liquid.

Scoring

The square plates were divided into 5 equally large areas. Area 1 had the highest concentration of salt, while area 5 had the lowest concentration (Figure 3). After 5 minutes of placing the worms on the plate, the worms in each area were counted from top to bottom and a chemotaxis index was calculated. This process was repeated 30 minutes after placing the worms. Area 3 was not considered in scoring, since worms were placed there artificially and leftover food from the transfer might affect the assay. The chemotaxis index was determined by adding up the individuals in area 1 and 2, together making up n(high), and adding area 4 and 5 together, resulting in n(low).

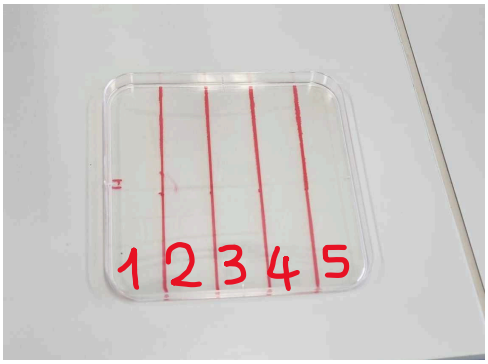

Figure 3: areas on plate.

Three rounds of assays were performed, experimenting with different factors and variables.

Week 1

The first week, worms without salt conditioning were used. We used a synchronized N2 and an unsynchronized N2 male plate on a gradient 24 hours after preparation. After this, a synchronised N2 and unsynchronized JU775 plate that was starving was placed on a gradient that dried for 48 hours.

Week 2

During this week, we started with salt conditions, a high salt, normal salt synchronized N2 and a high salt and normal synchronized JU775 plate were used on a gradient 24 hours after preparation.

Week 3

Lastly, eight non-synchronized plates were used. Four were different strains that had been raised on a standard salt plate (LKC34, DL238, CB4856, CX11314). The other four were starved combinations of high salt and standard salt plates of ED5017 and N2 with males. The last 4 plates were synchronized, two N2's with males on a high salt and normal plate and two MY16 X male N2 on a high salt and normal plate as well.

Analysis

All results to the questions were first analysed visually, whenever there was a noticeable difference, ANOVA was performed to determine the significance of the result.

Acknowledgements

First and foremost, I want to thank my supervisor Paris Veltsos for helping me every step of the way. He welcomed me in a very helpful and kind work environment where I felt truly supported. He lead me in the right direction and showed me the ropes of real scientific work, standing by me through ups and downs. Paris also taught me the basics of programming in R, in which all results of this study were analysed. I also want to thank my promotor Wen-Juan Ma for giving me the opportunity to work in her lab. I'm grateful to Maud Januarius for helping me with the lay-out of this paper. I want to give a special thanks to Professor Patrick Laurent for supplying us with the male N2 *C. elegans* strain and training us to distinguish males from females and adults from L4's during a lab visit, and the Liesbet Temmerman lab at KU Leuven for supplying the CeNDR strains used. Lastly, I want to thank my colleague Marziehsadat Bagheri for helping with maintenance and Ann Pauwels for preparing the agar we used to make the plates and gradients.

Bibliography

Henry Ancell, Andre Pires-daSilva, Sex-specific lifespan and its evolution in nematodes, Seminars in Cell & Developmental Biology, Volume 70, 2017, Pages 122-129, ISSN 1084-9521, https://doi.org/10.1016/j.semcdb.2017.05.012.

Elvin, M., Snoek, L. B., Frejno, M., Klemstein, U., Kammenga, J. E., & Poulin, G. B. (2011). A fitness assay for comparing RNAi effects across multiple C. elegans genotypes. BMC genomics, 12, 510. https://doi.org/10.1186/1471-2164-12-510

Fagan, K. A., & Portman, D. S. (2014). Sexual modulation of neural circuits and behavior in Caenorhabditis elegans. Seminars in cell & developmental biology, 33, 3–9. https://doi.org/10.1016/j.semcdb.2014.06.007

Gray, J. C., & Cutter, A. D. (2014). Mainstreaming Caenorhabditis elegans in experimental evolution. Proceedings. Biological sciences, 281(1778), 20133055. https://doi.org/10.1098/rspb.2013.3055

Girard, L. R., Fiedler, T. J., Harris, T. W., Carvalho, F., Antoshechkin, I., Han, M., Sternberg, P. W., Stein, L. D., & Chalfie, M. (2007). WormBook: the online review of Caenorhabditis elegans biology. Nucleic acids research, 35(Database issue), D472–D475. https://doi.org/10.1093/nar/gkl894

Hiroki, S., Yoshitane, H., Mitsui, H., Sato, H., Umatani, C., Kanda, S., Fukada, Y., & Iino, Y. (2022). Molecular encoding and synaptic decoding of context during salt chemotaxis in C. elegans. Nature communications, 13(1), 2928. https://doi.org/10.1038/s41467-022-30279-7

Khanna, N., Cressman, C. P., 3rd, Tatara, C. P., & Williams, P. L. (1997). Tolerance of the nematode Caenorhabditis elegans to pH, salinity, and hardness in aquatic media. Archives of environmental contamination and toxicology, 32(1), 110–114. https://doi.org/10.1007/s002449900162

Lamitina, S. T., Morrison, R., Moeckel, G. W., & Strange, K. (2004). Adaptation of the nematode Caenorhabditis elegans to extreme osmotic stress. American journal of physiology. Cell physiology, 286(4), C785–C791. https://doi.org/10.1152/ajpcell.00381.2003

Lim, J. P., Fehlauer, H., Das, A., Saro, G., Glauser, D. A., Brunet, A., & Goodman, M. B. (2018). Loss of CaMKI Function Disrupts Salt Aversive Learning in C. elegans. The Journal of neuroscience : the official journal of the Society for Neuroscience, 38(27), 6114–6129. https://doi.org/10.1523/JNEUROSCI.1611-17.2018

Luo, L., Wen, Q., Ren, J., Hendricks, M., Gershow, M., Qin, Y., Greenwood, J., Soucy, E. R., Klein, M., Smith-Parker, H. K., Calvo, A. C., Colón-Ramos, D. A., Samuel, A. D., & Zhang, Y. (2014). Dynamic encoding of perception, memory, and movement in a C. elegans chemotaxis circuit. Neuron, 82(5), 1115–1128. https://doi.org/10.1016/j.neuron.2014.05.010

Mah, M.W., Mitha, I., Trinh, A., & Wu, D. (2017). Effect of NaCl concentration on the mid-body movement of Caenorhabditis elegans. The Expedition, 6.

Peymen, K., Watteyne, J., Borghgraef, C., Van Sinay, E., Beets, I., & Schoofs, L. (2019). Myoinhibitory peptide signaling modulates aversive gustatory learning in Caenorhabditis elegans. PLoS genetics, 15(2), e1007945. https://doi.org/10.1371/journal.pgen.1007945

Sakai, N., Iwata, R., Yokoi, S., Butcher, R. A., Clardy, J., Tomioka, M., & Iino, Y. (2013). A sexually conditioned switch of chemosensory behavior in C. elegans. PloS one, 8(7), e68676. https://doi.org/10.1371/journal.pone.0068676

Teotonio, H., Carvalho, S., Manoel, D., Roque, M., & Chelo, I. M. (2012). Evolution of outcrossing in experimental populations of Caenorhabditis elegans. PloS one, 7(4), e35811. https://doi.org/10.1371/journal.pone.0035811

Watteyne, J., Peymen, K., Van der Auwera, P., Borghgraef, C., Vandewyer, E., Van Damme, S., Rutten, I., Lammertyn, J., Jelier, R., Schoofs, L., & Beets, I. (2020). Neuromedin U signaling regulates retrieval of learned salt avoidance in a C. elegans gustatory circuit. Nature communications, 11(1), 2076. https://doi.org/10.1038/s41467-020-15964-9

Weinberg, E. D. (1959). Gradient Agar Plates. The American Biology Teacher, 21(8), 347–350. https://doi.org/10.2307/4439185

Timothy A Crombie, Ryan McKeown, Nicolas D Moya, Kathryn S Evans, Samuel J Widmayer, Vincent LaGrassa, Natalie Roman, Orzu Tursunova, Gaotian Zhang, Sophia B Gibson, Claire M Buchanan, Nicole M Roberto, Rodolfo Vieira, Robyn E Tanny, Erik C Andersen, CaeNDR, the Caenorhabditis Natural Diversity Resource, Nucleic Acids Research, Volume 52, Issue D1, 5 January 2024, Pages D850–D858, https://doi.org/10.1093/nar/gkad887

Michael P. Gardner, David Gems, Mark E. Viney, Aging in a very short-lived nematode, Experimental Gerontology, Volume 39, Issue 9, 2004, Pages 1267-1276, ISSN 0531-5565, https://doi.org/10.1016/j.exger.2004.06.011.

Walsh, J. D., Boivin, O., & Barr, M. M. (2020). What about the males? the C. elegans sexually dimorphic nervous system and a CRISPR-based tool to study males in a hermaphroditic species. Journal of neurogenetics, 34(3-4), 323–334. https://doi.org/10.1080/01677063.2020.1789978

Teotonio, H., Carvalho, S., Manoel, D., Roque, M., & Chelo, I. M. (2012). Evolution of outcrossing in experimental populations of Caenorhabditis elegans. PloS one, 7(4), e35811. https://doi.org/10.1371/journal.pone.0035811