Differences in salt chemotaxis between Caenorhabditis species and sexes

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Abstract

Salt chemotaxis in Caenorhabditis nematodes is shaped by species, sex, and previous salt conditioning. We tested three species: *C. elegans*, *C. remanei*, and *C. brenneri* under high and low salt conditions for their salt preference. We documented the movement of male and female worms across a gradient plate. Using mixed-effects and ordinal logistic models we show that chemotaxis towards a particular salt concentration is not explained by species or sex alone, but by their interactions with the salt conditions in which the worms grew up in. Across models, interaction effects were more informative than single factors *C. remanei* males showed the strongest behavioral plasticity based on their developing salt conditions. This species-specific pattern may reflect biological differences.

Introduction

For over 50 years, *Caenorhabditis* roundworms have served as model organisms. Most notably *C.elegans,* a hermaphroditic species that could be kept in the lab. The foundational work on studying mutant phenotypes (Brenner *et al.* 1974) made it a popular choice for studies in the fields of genetics, development, pathology and behavior. The species carries many properties that made it a good choice for molecular studies. Adult *C.elegans* grow to 1 millimeter long and their tissues are translucent, making them easily observable by light microscopy. They have a short life cycle, generating many offspring after 3½ days (Brenner *et al.* 1973). They have an invariant number of somatic cells and an accurate description of all 302 hermaphrodite neurons has been made (White *et al.* 1976). More recently their entire connectome was described (Cook et al 2019).The eggs of different strains can survive freezing, allowing for easy distribution to research labs worldwide (Corsi *et al*. 2005-2018).

In addition to the well-characterized *C. elegans*, there are multiple described species within the genus. *Caenorhabditis* are prevalent colonizers of nutrient- and microorganism-rich organic material, like decaying plant matter. Unlike *C. elegans*, which is predominantly hermaphroditic, most others are gonochoristic species, like *C. remanei* and *C. brenneri*. While *C. remanei* inhabits northern temperate regions, *C. brenneri* is more circumtropically distributed. They are strongly tied to anthropogenic habitats and certain invertebrates, but many aspects of their ecology are still unknown (Sudhaus *et al*. 2007). Phylogenetic analyses place them to be closely related to *C. elegans*. They seem to have diverged at the same time, around 110 mya (Kionke *et al.* 2004; Cutter, 2008).

C. elegans uses its fundamental ability to perceive chemical signals to interact with its environment, adjusting its behavior accordingly. Chemotaxis behavior has a key role in *C. elegans* survival since it is used to forage for food, find mates, develop properly, and avoid pathogens and toxic substances (Bargmann *et al*. 2006). They possess neurons that penetrate the cuticula to expose their sensory cilia to the environment (Ward *et al.* 1975). The neurons responsible for salt chemotaxis initiation are a (left-right) symmetric neuron pair, called Amphid Sensory Neurons of subtype E (ASE). ASEL is primarily sensitive to sodium, whereas ASER is primarily sensitive to chloride and potassium (Pierce-Shimomura *et al*. 2001). The animal movement over gradients has been characterized to follow a simple strategy. Short-term memory of an attractant concentration is used to decide whether to continue moving in the same direction or pivot to another (Pierce-Shimonura *et al*. 1999). Due to sensitivity to different chemicals, the ASEL neuron will be activated by increases in salt level and ASER will activate by decreases in salt level (Shindou *et al*. 2019). Salt chemotaxis is plastic and can be influenced by multiple factors. It is strongly linked to food foraging through associative learning. *C. elegans* is repelled by high salt concentrations when grown at low salt concentrations with food abundance or when grown at high salt concentrations under starvation conditions (Rahmani *et al.* 2021). This short-term conditioning response is known to persist for 30 minutes (Kano *et al.* 2008).

To our knowledge, there are no published results on salt chemotaxis behavior in *C. brenneri* and *C. remanei*. Our lab's interest in sexually antagonistic genes and the ease of use of *Caenorhabditis* nematode methods drove us to set up this experiment, with special interest in the differences in chemotaxis behavior between species, sex, and their interactions. As research question we stated: "Is there a significant difference in salt chemotaxis, when comparing species, sexes and their interaction across salt conditioning treatments?". A previous study in the lab, by Tristan Cornelis (2024; unpublished) reproduced the literature findings in *C. elegans* and inspired the methods applied to this study.

We did not have a clear expectation whether different species would respond to salt differently. On one hand they share many anatomical and molecular features and the plasticity in salt preference is likely equally important for all of them, so they would not differ. On the other hand, they inhabit distinct geographical areas, associate with different invertebrates and were collected from different habitats which might differ in salt concentration, so that particular species or strains might have a different default salt preference. For sex, we might expect a bigger difference between males and females in the gonochoristic *C. brenneri* and *C. remanei* than in *C. elegans* for multiple reasons: First, *C. elegans* are mostly hermaphrodite with males occasionally arising by meiotic mutations (Corsi *et al*. 2005-2018). The male-hermaphrodite behavioral difference might therefore be smaller than the male-female difference of gonochoristic species. Second, *C. elegans* males lack many characteristics of males of closely related species, such as the ability to induce paralysis in females that helps in mating (Garcia *et al.* 2007). One might expect that the more differentiated males of gonochoristic species might also display more behavioral differences, such as stronger wandering behavior which might affect the salt chemotaxis measured here.

Material and Methods

Worm line origin and maintenance: The standard N2 *C. elegans* strain was provided by the lab of Patrick Laurent (ULB, Belgium). Multiple *C. remanei* and *C. brenneri* strains were kindly provided from the lab of Marie-Anne Felix (Université Paris, France) who holds an extensive worldwide collection of *Caenorhabditis* strains. The strains were chosen to represent multiple worldwide locations and habitats of origin (Appendix 3, figure A1). All lines were maintained on standard NGM medium at 18 ºC, 50% humidity and 12 h light-dark cycle, and transferred 2-3 times every week by chunking to new NGM plates that had been seeded with *Escherichia coli* of the OP50 strain bacteria for at least 24 h. NGM is prepared by adding 1.7 w% Agar, 0.3 w% NaCl, 0.25 w% peptone to water. After autoclaving, the mixture is kept at 55°C for at least two hours, until cooled down. Then, 0.1 v% cholesterol (5mg/ml, dissolved in ethanol), 2.5 v% K_2HPO_4 (2 M, at pH = 6), 0.1 v% $CaCL_2$ (1 M) and 0.1 v% $MgSO_4$ (1 M) are added and the medium can be poured. High-salt NGM is produced in the same way, but adding 0.6 w% NaCl instead. In a few cases, high-salt NGM was used for worm maintenance.

Worm synchronization produces a large population of worms that hatched at the same time and is used to minimize variance in experiments. Synchronized worms were obtained by purifying eggs out of an NGM petri-dish with worms and then placed on a fresh NGM petri-dish. The worms were washed off the plate with 2 ml of M9 solution and put in a 15 mL Falcon tube to which 13 ml demineralized water was added. M9 is a mixture of 0.58 w% Na_2HPO_4 (Anhydrous) 0.3 w% KH_2PO_4 (Anhydrous) 0.05 w% NaCl and 0.1 g NH_4Cl in ddH_2O, which is autoclaved. The tubes were centrifuged at 500 × RCF for 3 minutes. The supernatant was removed using a P1000 pipette, down to 1.5 ml. Then, 2 ml of bleach (1.25 ml 10 M NaOH to every 5 ml of NaClO (6-14% active chlorine (Merck))) was added. After 4.5 min, eggbuffer was added to the 15 ml mark. Eggbuffer is a mixture of 12.5 mL HEPES at 1 M, 29.5 ml NaCl at 2 M, 12 mL KCl at 2 M, 1 ml $CaCl_2$ at 1 M and 1 ml $MgCl_2$ at 1 M in ddH_2O. The tubes were centrifuged at 500 – 1000 × RCF for 3 min. After removing the supernatant to 1.5 ml whilst avoiding the floating eggs, eggbuffer was added to the 10 ml mark and the tubes were centrifuged once more at 500 – 1000 × RCF for 3 min. The supernatant was again removed to 1.5 mL and the pellet was resuspended with the addition of 1 ml M9. Lastly, the liquid was transferred to unseeded NGM of normal and high salt concentrations, with bacterial lawns that had been growing for at least 24 hours at room temperature.

Salt gradient plates were prepared by consecutively pouring two NGM media with different NaCl concentrations over each other in a square 12 × 12 cm petri dish. First, the high-salt NGM and normal NGM were prepared in 1 l flasks and kept at 55 ºC. The petri dishes were tilted by elevating one side with coin stacks or rubber blocks with a total height of 7 mm. The point of contact was positioned two centimeters from the elevated edge of the plate. This resulted in a slope of approximately 4º. A picture of the setup is provided in Figure 1A. Seventy mL of the high-salt agar were added and allowed to set for 5 min. Then, the elevating supports were removed, and 70 ml of the low-salt agar was poured on top, ensuring it lies flat. The gradients were prepared 24-72 h before being used in the experiment, allowing the salt to diffuse to the top layer of agar, effectively creating a linear salt gradient, as in Weinberg *et al.* 1959.

Salt preference was measured by putting the synchronized worms along the central-line of the gradient plates and observing their movement in relation to the gradient. First, lines were drawn on the back of the gradient plates, every 2 cm, to split them in 6 zones of different salt concentration (Figure 1B). Synchronized worms were transferred by washing them from their plates using 1 ml of M9, and pipetted onto the center line of the gradient plate. In a few cases the worms were plated after centrifugation at 500 × RCF, removal of the supernatant and resuspension in 1mL of M9 in an attempt to remove bacterial contamination, however this was not successful.. Excess liquid was removed using paper towels, allowing the worms to immediately move freely. Using this method the worms were distributed over the center two zones (3 and 4).

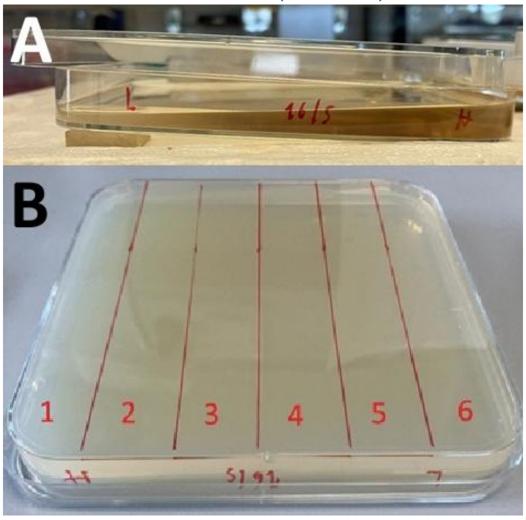

Figure 1: Steps in gradient preparation **A.** Tilted plate setting with high salt agar. **B.** Gradient with labeled zones (1-6), ranging from highest to lowest salt concentration respectively.

After 30-90 min the worms in the highest and lowest two zones were counted under a Leica stereoscope. We measured the salt preference on 100 different gradient plates. Practical issues led to omitting of some datapoints. These included plate contamination which interfered with free, unbiased, worm movement (3 cases), plates that used unsynchronized worms (3 cases) and when the gradients were made out of the wrong medium (2 cases).

The **salt chemotaxis index** was calculated as in (Kano *et al*. 2008), by adding the worms that moved towards the higher salt concentration (zones 1 and 2) and subtracting those that moved in the opposite direction (zones 5 and 6) from it. This number was then divided by the number of worms present in the four counted zones. The worms in the center two zones were not counted, as they were considered not to be attracted to high or low salt. For *C. remanei* and *C. brenneri* we calculated separately an index for males and females. We counted the *C. elegans* hermaphrodites as females. We kept data on the age of the maintenance plate used for synchronization, and the experiment date. We noted the time on gradient, prior worm centrifugation and salt concentration (high or low salt) of the plate worms developed after synchronization .

For the **statistical analysis** and visualization of the data we used R version 4.5.0 (R core team 2025) and ggplot2 version 3.5.2 (, package. The structural errors (introduced by not synchronizing worms, contaminated gradients and use of non NGM) were removed, to improve the reliability of the results. The data collected on 7-05-2025 deviated markedly from the pattern across other replicates. These datapoints were removed from further visualization and analysis with graphical and statistical justification (Appendix 1). After removal of structural errors (8), and the 7-05-2025 data (21), 71 high quality plates remained for statistical analysis. We visualized the effects of all recorded variables on the chemotaxis index. As the main focus of the experiment was to study the response of worms, conditioned by salt concentration , every plot included this information. All direct visualizations of chemotaxis index in function of different factors (salt treatment, species, strain, sex, gradient age, worm age and observation time) are provided in Appendix 2. When graphically justified, the student t-test was used to assess whether their impact was significant on the chemotaxis index or not. The effect of sex was assessed using a paired t-test. Furthermore, we visualized the proportion of worm movement to the different zones (Figure 2).

We fitted different statistical regression models to explain the behavior of worms on the salt gradients focusing on the effects of salt treatment, sex, species, and their interactions. Ordinal logistic regression provides the most granular view, capturing not just whether attraction occurred but to what degree, by predicting the probabilities for worms moving to every zone. Meanwhile, a mixed model accounts for repeated or hierarchical structure in continuous responses.

First we fitted a linear mixed-effects model with species, sex, salt treatment and their interactions as fixed effects, and gradient plate and experiment date as random effects. Preliminary analysis of observation time, maintenance plate age, worm age and centrifugation showed them to be unimportant so they were excluded from the final analysis (Appendix 2). We didn't have enough reliable data to research the effects of strain accurately; therefore the effect of strain was also left out. We used the DHARMa package version 0.4.7 (Hartig 2024) to assess model diagnostics via simulated residuals, allowing us to check for deviations from model assumptions such as dispersion, zero-inflation, and non-uniformity using a simulation-based approach. We dropped interactions and terms if they were not significant, and justifiable for removal using Likelihood Ratio Tests (LRT's) until we reached a minimal adequate model with significant terms at the 5% level. We checked model diagnostics again with the DHARMa package.

As logistic regressions don't handle the missing data of male *C. elegans* well we decided to fit an ordinal logistic regression model excluding the factor sex, one excluding all *C. elegans* data and a last model combining the factors sex and species into a new variable. We removed the plate C3 from our analysis as it influenced the models disproportionately and was an outlier to the expected response. Once we achieved the minimum adequate models with factors at the 5% level we performed Brant tests, with the brant package version 0.3.0 (Schlegel *et al.* 2020),to see if the model assumption of having proportional odds was upheld, and thus if ordinal logistic regression was appropriate.

Results

Our simple t-tests (Appendix 2) showed that, as expected (Corsi, 2005-2018 & Tristan), salt treatment had a significant effect on chemotaxis index. We detected no significant differences in chemotaxis index across species or sex.

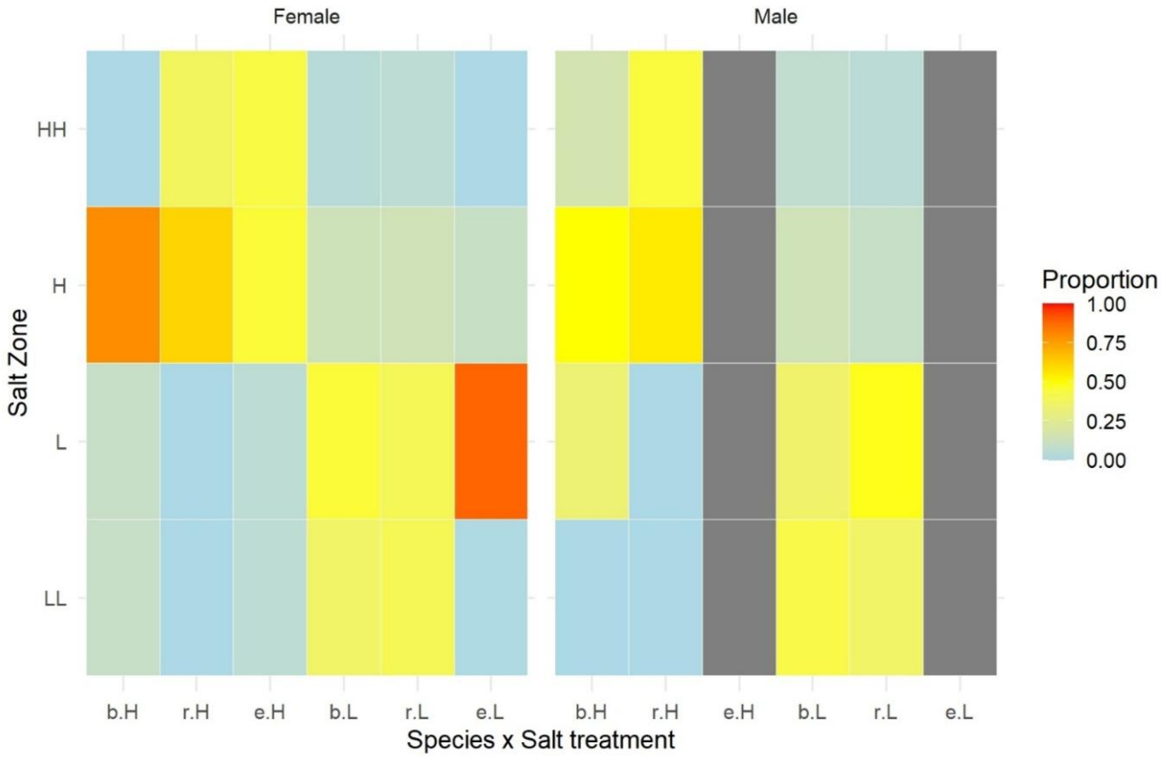

Figure 2: Heatmaps of worm movement, the proportion of males and females across increasing salt concentration zones (LL, L, H, HH) in three *Caenorhabditis* species (*C. brenneri* [b], *C. remanei* [r], and *C. elegans* [e]). Data are grouped by salt treatment (High [H] or Low [L]).

For the **linear mixed model**, with all interactions included, we found the random effect tag to explain 75.2% of the variation in our data, while date explained only 8.3%. However, as tag only has one or two index values per category, it's just overfitting the data. A LRT also suggested a model without the random effect of tag made no significant difference for the model, so we removed it from our model. We found a significant difference between salt treatment with a borderline significant interaction between sex and salt treatment. Residual diagnostics using the DHARMa package revealed significant deviations from uniformity (KS test: $p < 0.05$), with notable quantile imbalances. These results suggest potential model misspecification or distributional mismatch. We removed the fixed effect of species from the model altogether, as the factor did not significantly improve the model. Our minimal adequate model contained sex and salt treatment as fixed effects and date as a random effect, explaining 21% of all variation. The predictors for the main effects are reported in Table 1 under the linear mixed regression column. We found significant sex to salt treatment interaction, indicating males behavior is more plastic. We found borderline ($p = 0.052$) significance for sex impacting chemotaxis. Model diagnostics for the QQ plot and overall residual uniformity tests indicated an acceptable model fit. However, a significant deviation from uniformity was still detected within groups, suggesting some residual heterogeneity at the group level. This reflects some unmodeled variation is present, but other diagnostics did not indicate major violations. This was probably caused by the missing data for male *C. elegans*. The model is considered appropriate with caution regarding within-group variability.

Predictors and p-values for the **ordinal logistic model** excluding the effect of sex are summarized in Table 1and predicted proportions are plotted in Figure A14. The effect of salt treatment was significant. We found an interaction between species and salt treatment with *C. remanei* having a higher index than the other two species. Furthermore we found the factor species makes a significant effect in *C. elegans* compared to *C. brenneri*. In addition *C. elegans* had a stronger response than the other two species. The Brant test suggested model assumptions were not violated.

Predictors and p-values for the ordinal logistic model excluding the *C. elegans* are summarized in Table 1. The predicted proportions plot is shown in Figure A15. We found a significant three-way interaction between sex, species and salt treatment. Two-way interactions between salt treatment and species or sex were also significant, as were the factors salt treatment and sex. Species was not significant. A Brant test proved model assumptions were not violated.

The **ordinal logistic model using the transformed variable sexspecies** is summarized in Table 1. It showed significant interactions between salt treatment and sexspecies for the male *C. brenneri*, and both groups of *C. remanei*. For this reason we didn't simplify the model. The Brant test showed the model assumptions were respected.

Table 1: Summary of model predictors with their p values and AIC of all performed regressions.

Predictors	*Intercept*	*Linear mixed regression (1)*	*Ordinal logistic regression without sex (2)*	*Ordinal logistic regression without C. elegans (3)*	*Ordinal logistic regression using sexspecies (4)*	*Predictors (and intercept) for sexspecies model*
Salt treatment	High	-0.8271 ($p< 0.001$)	-1.447 ($p < 0.001$)	-1.177 ($p<0.001$)	*C. brenneri* males: 0.655 ($p = 0.036$), *C. elegans* hermaphrodites: 0.887 ($p < 0.001$), *C. remanei* females: ns *C. remanei* males: 1.095 ($p <0.001$)	Sexspecies (*C. brenneri* females)
Sex	Female	0.3029 ($p = 0.052$)	Not in model	0.642 ($p = 0.038$)		
Species	*C. brenneri*	Removed, ns	*C. elegans*: 0.624 ($p < 0.001$) *C. remanei*: ns	*C. elegans*: data removed *C. remanei*: ns		
Salt treatment – Sex interaction	High - Female	-0.4839 ($p = 0.020$)	Not in model	-0.745 ($p= 0.042$)	-1.169 ($p < 0.001$)	Salt treatment (high)
Salt treatment – Species interaction	High – *C. brenneri*	Removed, ns	*C. elegans*: ns *C. remanei*: 0. 438 ($p = 0.036$)	*C. elegans*: data removed *C. remanei*: 0.920 ($p < 0.001$)	*C. brenneri* males: -0.757 ($p = 0.039$), *C. elegans* hermaphrodites: ns, *C. remanei* females:0.908 ($p<0.001$) *C. remanei* males: -0.972 ($p <0.001$)	Sexspecies – salt treatment interaction (*C. brenneri* females – high)
Sex – Species interaction	Female – *C. brenneri*	Removed, ns	Not in model	*C. elegans*: data removed *C. remanei*: ns		
Salt treatment - Sex – Species interaction	High – Female – *C. brenneri*	Removed, ns	Not in model	*C. elegans*: data removed *C. remanei*: -0.102 ($p = 0.010$)		
Akaike Information Criterion		16,160.5	16,160.502	4,629.927	16,092.9	

Discussion

Across the different models we found significant interaction terms. We first discuss the third model, in which a significant three-way interaction between salt treatment, sex, and species (comparing only *C. brenneri* and *C. remanei*) was found. This indicates that the effect of high salt treatment on salt attraction varies depending on both sex and species. The effect on the response of one species-sex group when switched between the different salt treatments cannot be explained by adding the independent effects of species, sex and salt treatment alone. This implies a species and sex-specific behavior change to salt between these two gonochoristic species. We can however generalize the effect of species or sex independently when comparing these two species, because when looking at the two-way interaction terms we notice the sex-species interaction term to be non-significant, but the other two are. Their predictors are not too different in absolute value, but are by far more impactful than the three way interaction term. This indicates that both sex-salt treatment as species-salt treatment interactions drive the biggest differences in behavior between these two species. From the effect of species being non-significant we can deduce that variation in response across the two species depends on the salt treatment we are observing. When conditions change from high to low salt, *C. remanei* shows a smaller decrease in salt attraction. We can describe the difference between sexes as males seeking the high salt conditions stronger under high treatment, but having a larger shift in behavior when conditions change. In essence, males show a more plastic behavior in the two species.

The second model fits it's data significantly worse than the third model does. This is not surprising, as a lot more data and thus more variation is introduced through adding the third species. In addition, this model is much simpler, restricting fitting as well. We cannot easily tell which model better describes salt chemotaxis in nematodes, but lean towards the values of the third model, due to the higher complexity in factors. In agreement with the last model, this model finds a species difference between *C. brenneri* and *C. remanei*, that is influenced through the interaction with salt treatment alone. In contrast, *C. elegans* seems to have a strictly different behavior than the other two species. It's attraction to salt is greater under every salt condition, having no significant interaction with salt treatment. As this model did not carry sex as a factor, it did not discriminate males from females. The effect we see between the species likely also carries the effect of having different sex distributions.

The fourth model revealed a nuanced and consistent pattern with our previous findings. Notably, male *C. remanei* and male *C. brenneri* both showed significantly reduced attraction to salt under low salt conditions, supporting the evidence that males of these species exhibit greater behavioral plasticity in response to prior salt exposure. In contrast, female *C. remanei* displayed the opposite pattern, with increased attraction under low salt, highlighting sex-specific strategies within the same species. *C. elegans* females maintained consistently high salt attraction regardless of treatment, aligning with earlier models that showed no significant interaction between species and salt in this species. This is in contradiction with literature findings. We attribute this discrepancy to two possible reasons. One possible explanation for the unexpectedly strong salt attraction in *C. elegans* is a structural difference in experimental conditions. Synchronization of *C. elegans* consistently yielded more individuals compared to the other species, which resulted in higher animal densities during conditioning and testing. This could have led to mild starvation conditions, altering chemotaxis behavior. We could run the models again, including the data gathered by Tristan Cornelis to check if this holds true. When we compare this model to the second model, that left out the effect of sex, we see noticeable shifts towards the interaction terms weighing more than the independent terms. We cannot directly compare the AIC of this model with others, as it uses different predictors. The combined findings for this model support the view that salt attraction behavior in nematodes is both species- and sexdependent, with males generally showing more treatment-sensitive responses. Modeling sex and species as a composite factor provided a simplified framework that reinforced the key interactions found in our more complex models, while allowing direct comparison of biologically meaningful subgroups.

When interpreting the mixed effects linear model, we have to be cautious in comparing it with the other models, as their response variable is different and we diagnosed deviations from uniformity. The model agreed on there being salt treatment – sex interactions and (almost) significant sex and salt treatment differences. Calculating chemotaxis index per gradient provided an elegant way to visualize and interpret the data, especially preliminary inquiries about methodology. While in literature it has been useful to visually compare treatments with completely different outcomes, it's use in capturing subtle differences in behavioral patterns, such as those related to species and sex, are outmatched by quantitative statistical methods.

Our data did not allow us to make any conclusions about strain differences, as our data really focused on one strain within each species. This was a decision we made to gain enough data to show species and/or sex differences, if any. We did not exclude the data strains with very few datapoints for the reason that they might capture some natural variability, even with their limited data. However, in this way species differences might have been severely affected by strain differences, which is a nested variable we didn't include in any model. Future studies should aim to robustly assess strain differences with thorough sampling of their natural environments.

Things we did not cover in our analysis, but might still yield interesting results are data sensitivity analyses. We could omit the data from all strains except for the well studied ones. Contrarily we could add outlier data, like from the removed date and see if our models are robust to these introductions or withdrawals. We also didn't describe or quantify variance in response differences between sex and species. Experimental next steps would be to repeat the experiment, whilst paying more attention that the methods are consistent. Especially the effects of starvation and contamination should be better controlled for. We could test for the effects of strains and even test strain crosses or intra-species crosses. Testing male *C. elegans* would be possible, however this would shift the focus from modelling natural conditions to understanding genomic influences.

Conclusion

Salt chemotaxis in *Caenorhabditis* nematodes is influenced by the interplay of species, sex, and salt conditioning. Their behavior is not solely defined by main effects, but is significantly shaped by interaction terms.

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Appendix 1: justification for removing 7-05-2025 from model analyses, and probable cause of the structural error

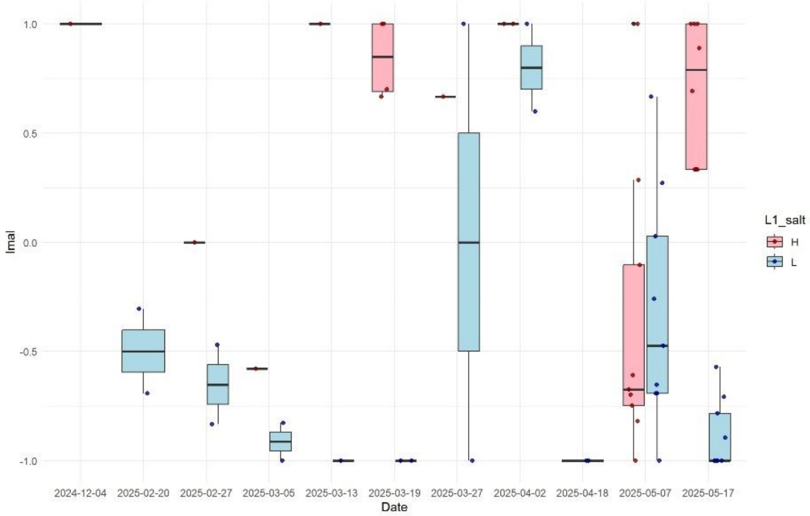

Figure A1: Male chemotaxis index by date, showing abnormal data for 07-05-2025.

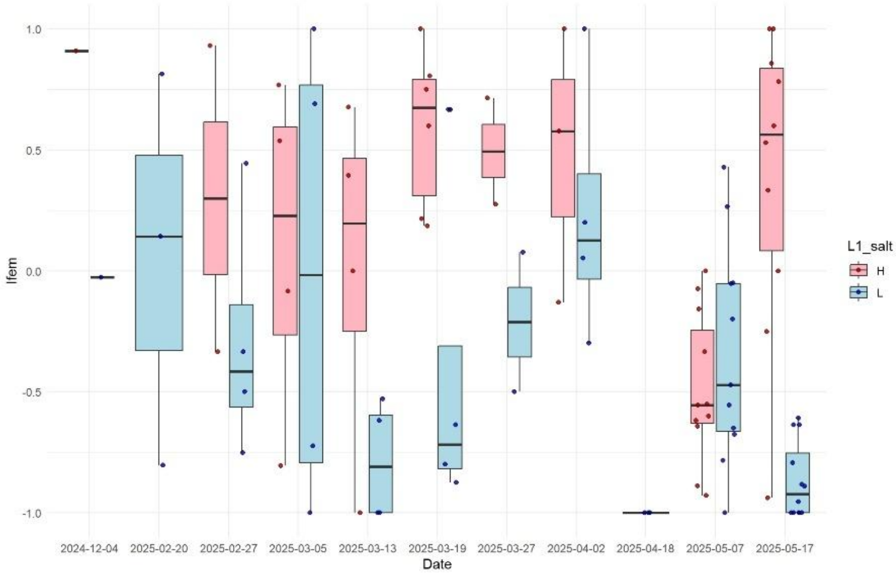

Figure A2: Female chemotaxis index by date, showing significantly abnormal data for 07-05-2025.

When we look at the indexes on 7-05-2025 we notice the index for the worms conditioned at high salt showed equal, if not stronger aversion to salt than those conditioned with low salt. This resembles the pattern of worms reacting to starvation under high salt conditions. We kept some data on the synchronized worms. Most of them were synchronized 7 days before the experiment, and showed negative indexes all around, for high salt treatments. Some were synchronized only 5 days before the experiment. Those yielded mixed results for high treatments. This strengthens the hypothesis that the aversion was caused by starvation. Presumably we added too little bacteria to the plates, or used the wrong bacterial culture in the conditioning of the worms. We checked if the deviance was significant using student t-tests for both indexes (Table A1). It proved significant for female index but not for male index. Due to there being more female data, by having 4 gradients with *C. elegans*, it makes sense the female data shows a more significant deviance from the other data. As the graphs looked to portray the same effect in males as it did for females, we decided to omit the data from this date.

Table A1: Student t-test results for difference in female and male indexes of the data collected on 07-05-2025 vs all other data

	Female index	Male index
t-value	2.7059	1.5099
df	67.416	46.624
p-value	0.00862*	0.1378

Appendix 2: direct effect plots and comparisons with chemotaxis indexes

Treatment differences

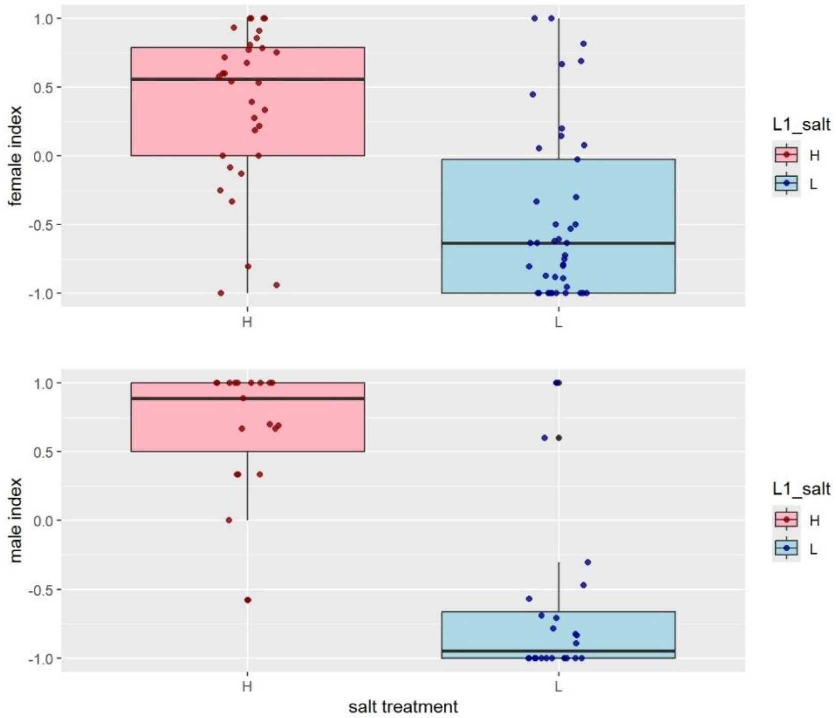

Figure A3: Visualisation of male and female index by salt treatment group

Table A2: results of t-tests testing for significant differences between salt treatments in male and female chemotaxis indexes.

	females	males
t-value	4.596	5.2635
df	86.549	53.565
p-value	1.456e-05*	2.548e-06*

Species differences

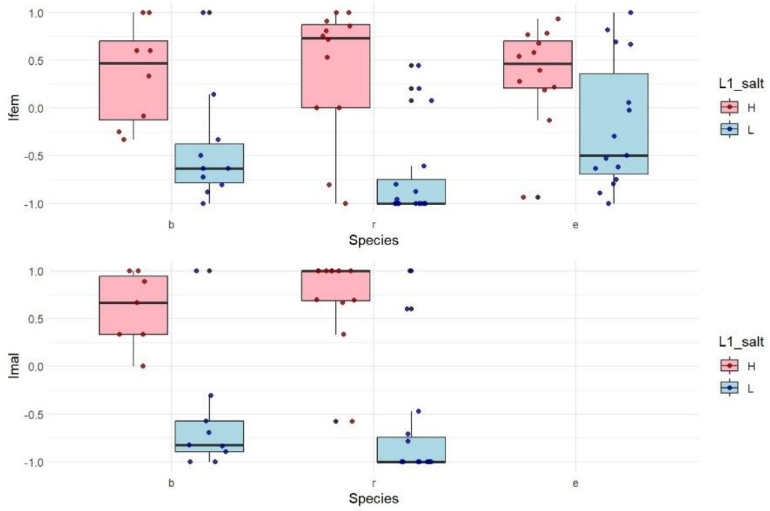

Figure A4: results in female (Ifem) and male (Imal) index, grouped by sex and species.

Table A3: results of t tests of t- tests testing for significant differences between species-sex groups.

	brenneri females	brenneri males	remanei females	remanei males	elegans females
t-value	-0.59973	-0.5502	1.722	0.5502	-1.3183
df	37.189	39.287	84.869	39.287	60.743
p-value	0.5523	0.5853	0.08872	0.5853	0.1923

Strain differences

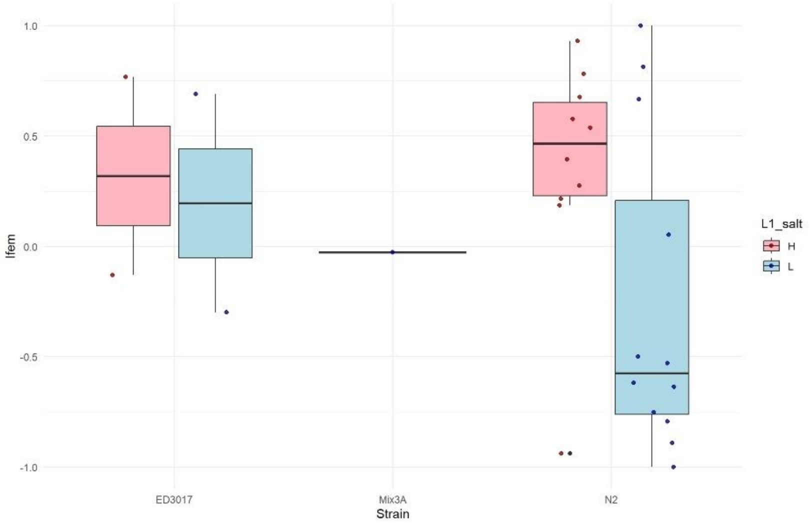

Figure A5: visualization of female index (Ifem) by strain for all *C. elegans* data.

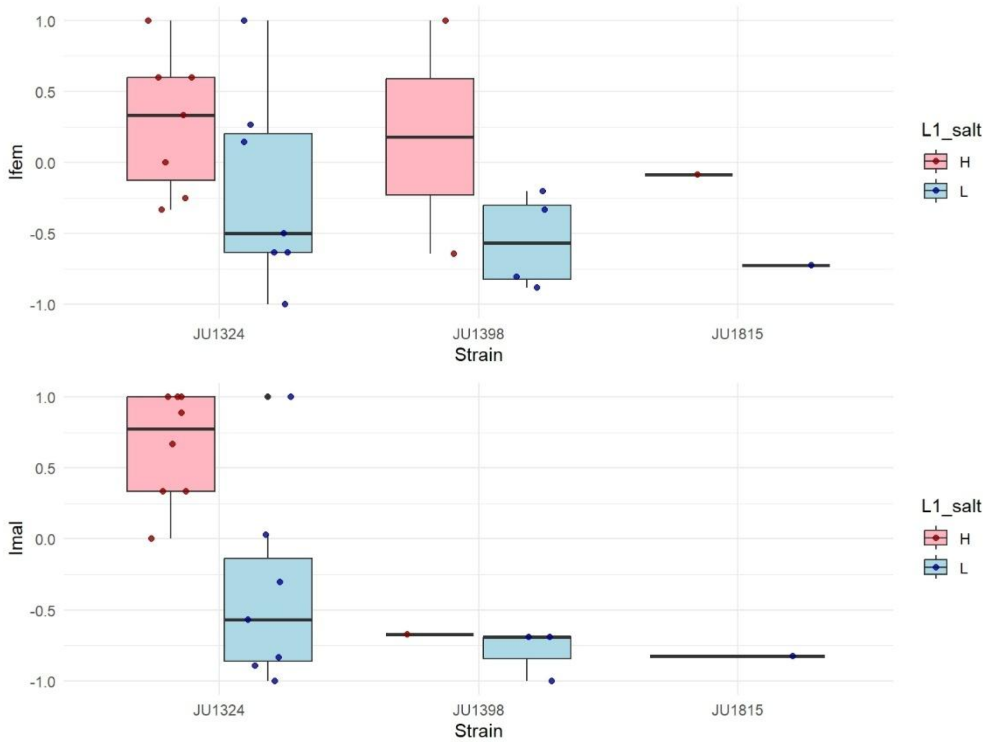

Figure A6: visualization of female index (Ifem) and male index (Imal) by strain for all *C. brenneri* data.

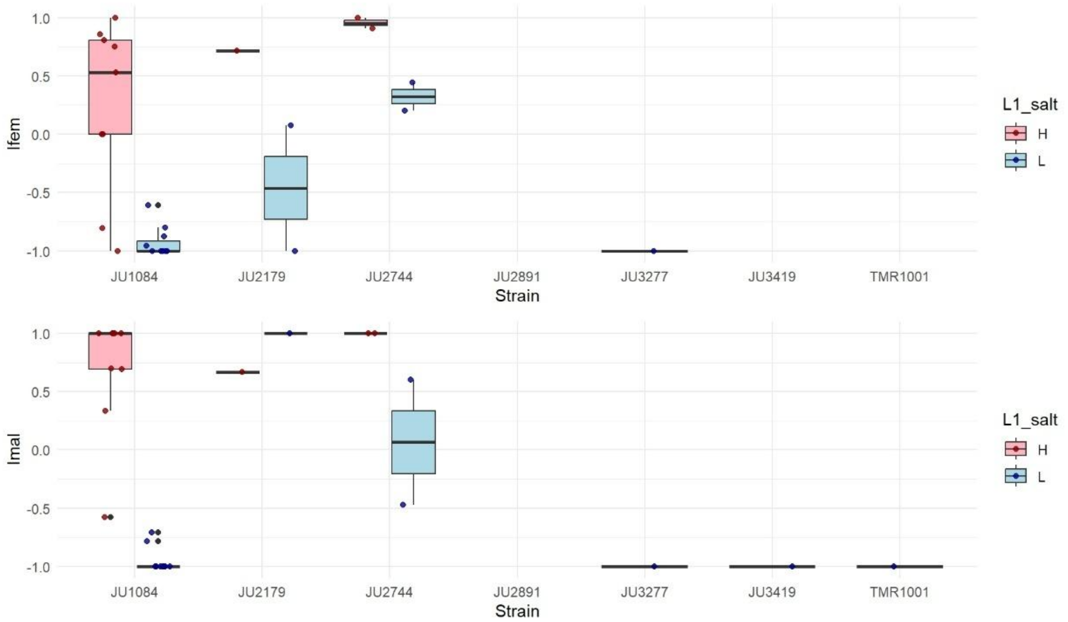

Figure A7: visualization of female index (Ifem) and male index (Imal) by strain for all *C. remanei* data.

Sex differences

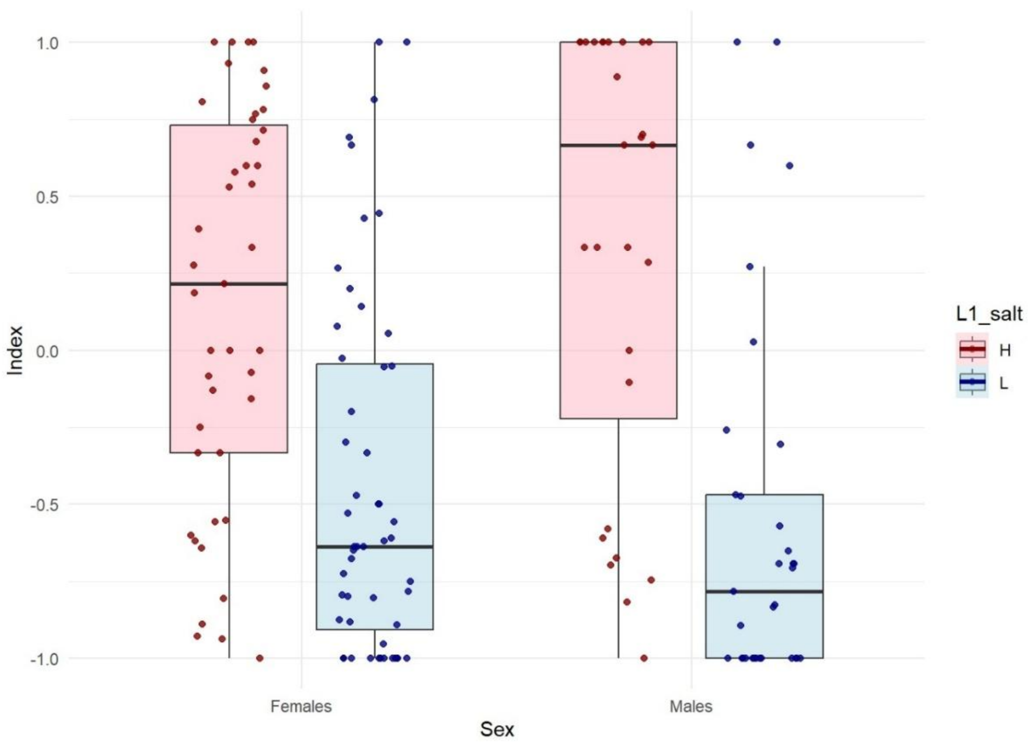

Figure A8: index comparison between the two sexes, by salt treatment.

Table A4: results of a paired t- test, testing for significant differences between females.

	Females vs Males (F intercept)
t-value	0.98404
df	56
p-value	0.3293

Effect of gradient age

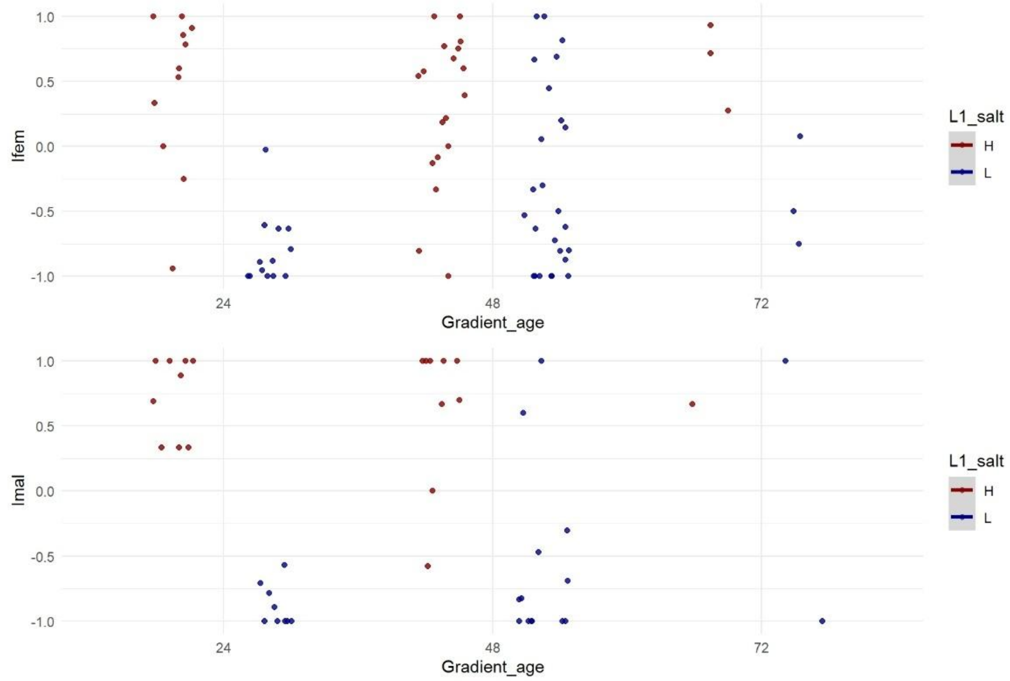

Figure A9: Scatterplot of female and male indexes in function of the gradient age.

Effect of time between putting on the gradient and observation

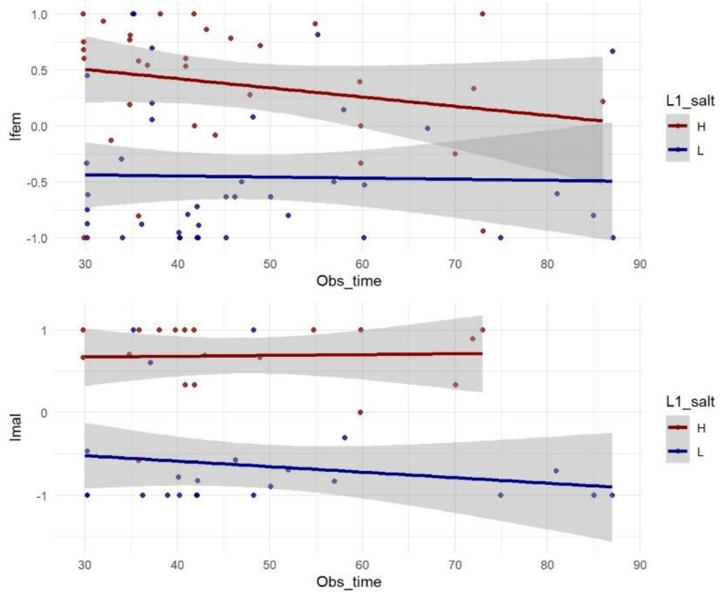

Figure A10: Scatterplot of female and male indexes in function of the observation time, with linear regression.

Effect of synchronized worm age

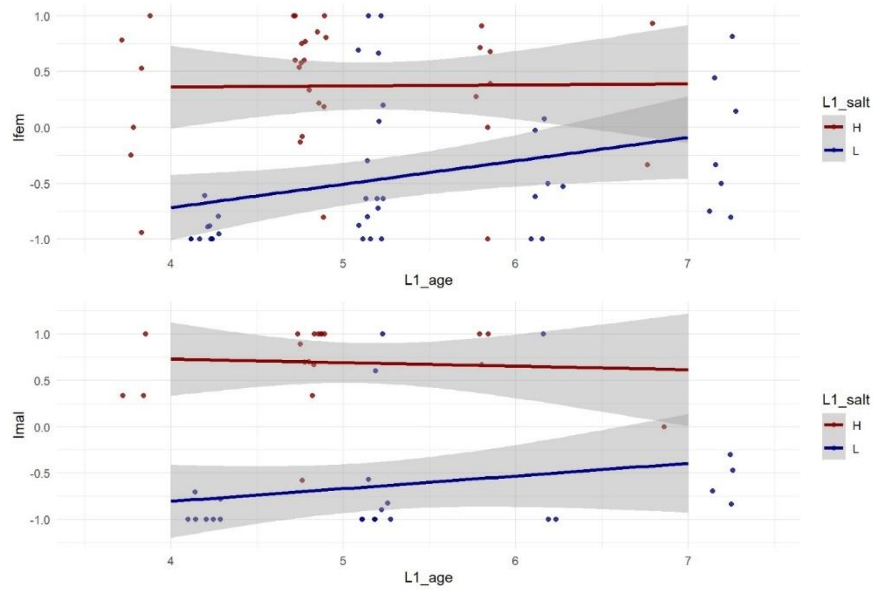

Figure A11: Scatterplot of female and male indexes in function of the worms age, with linear regression.

Interaction between male and female indexes

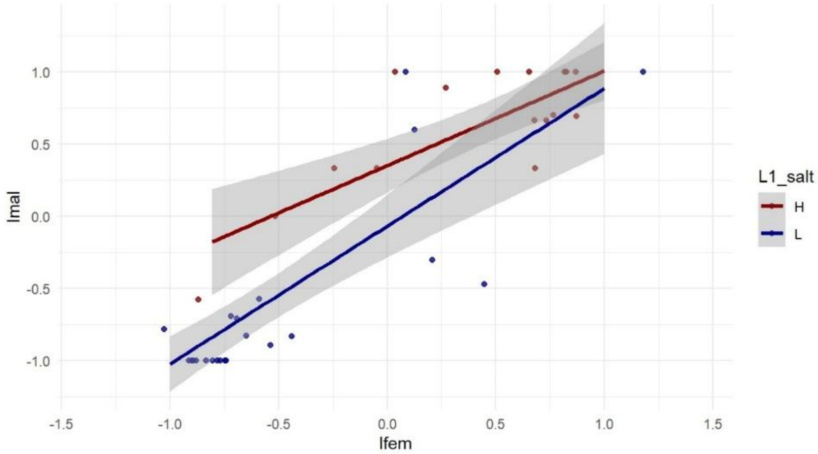

Figure A12: male index in function of female index, with linear regressions.

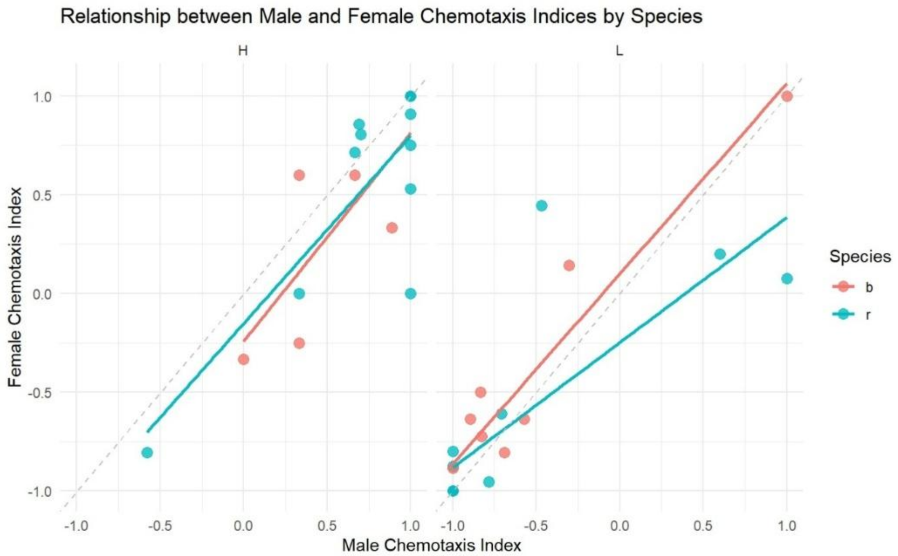

Figure A13: Female index in function of male index, grouped for high and low treatment worms, colored by species.

Appendix 3: Additional information about the examined worms

Table A5: Information about the origin of isolation for every used strain, by species.

Species	Strain	Origin
C. elegans	N2	England, Bristol, urban garden
C. elegans	ED3017	England, Edinburgh, urban garden
C. elegans	JT11398	USA, Seattle, urban garden
C. elegans	MY16	Germany, Münster, urban garden
C. remanei	TMR1001	Germany, Middelsachsen, Praebschuetz, rural garden, rotting apple
C. remanei	JU1086	Japan, Kakegawa, forest, vegetal litter, leaves/bark/soil
C. remanei	JU1084	Japan, Kakegawa, forest, vegetal litter, decomposing fruits/vegetables
C. remanei	JU2179	Canada, Montreal, rotting fruit, Juglans nigra
C. remanei	JU2558	France, Illkirch, rotting apple
C. remanei	JU2744	Korea, Seoul, rotting fruit, pumpkin?
C. remanei	JU3277	Czechia, Prague, rotten fruit, pear
C. brenneri	JU1324	India, Poovar, rural garden
C. brenneri	JU1398	Colombia, Medellin, rural garden
C. brenneri	JU1855	French Guiana, Comte valley, forest, rotting fruit

Appendix 4: visual representations for the ordinal logistic regression predictions

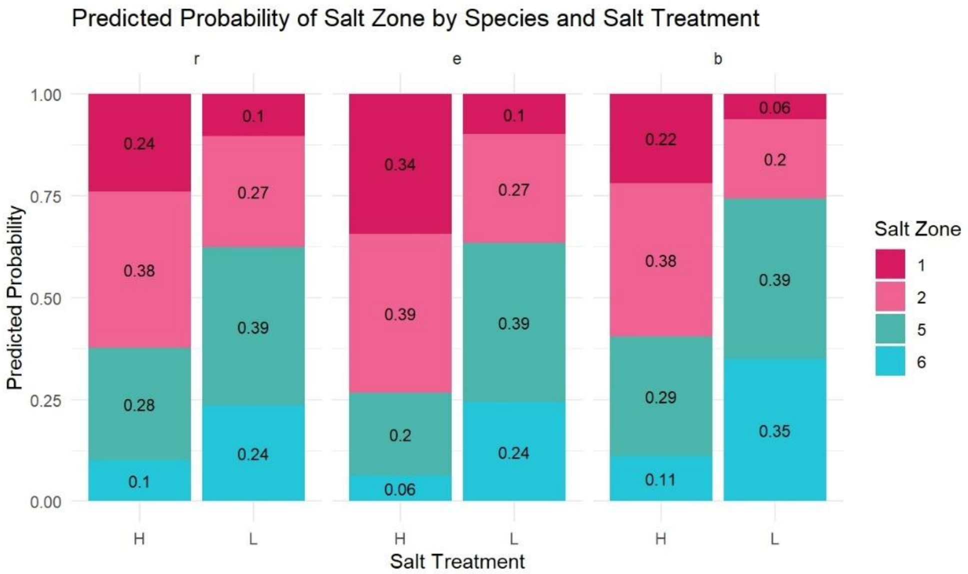

Figure A14: Predicted proportions plot of salt zone by species (C. remanei [r], C. elegans [e] and C. brenneri [b] at the top of the graph) and salt treatment (high salt [H] and low salt [L] on the bottom). The colors indicate the gradient plate salt zones (1, 2 are high; 5,6 are low).

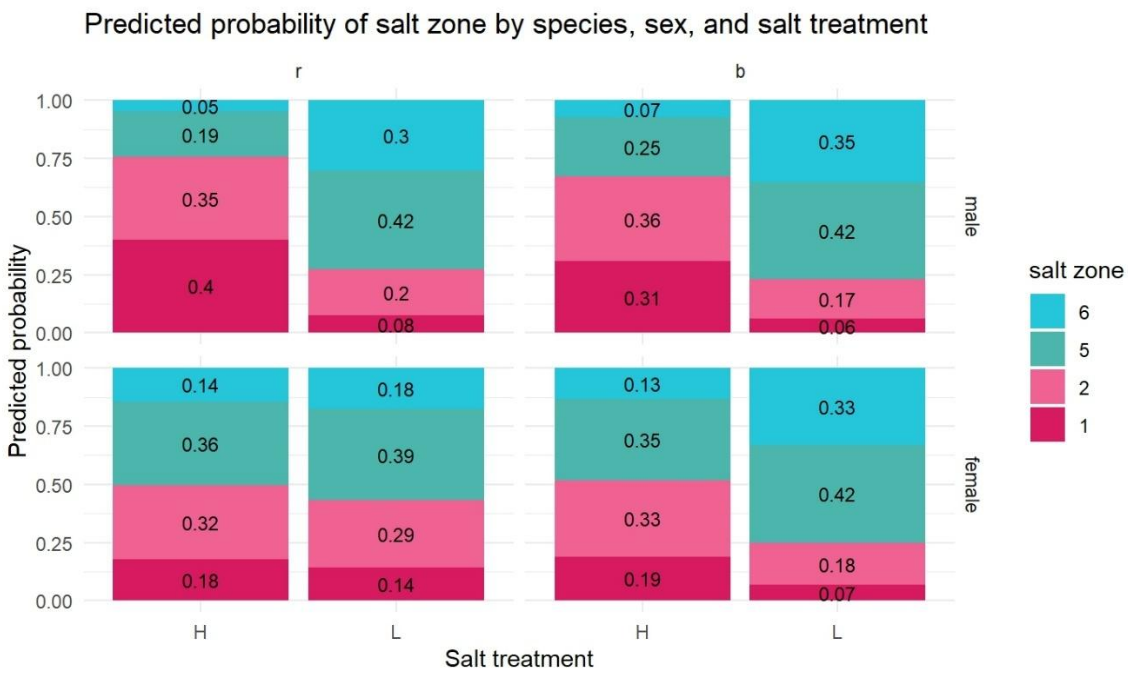

Figure A15: Predicted proportions plot of salt zone by species (*C. remanei* [r] and *C. brenneri* [b] at the top of the graph), salt treatment (high salt [H] and low salt [L] at the bottom of the graph), and sex (male and female on the right side of the graph).