The response of intra and interspecific hybrids to selection for high and low salinity in Caenorhabditis

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Abstract

In this study, we exposed Caenorhabditis populations generated from within and between species cross to different salt concentrations. After evolving six generations, we tested for long-term adaptation and changes in salt preference. We further determined reproductive output success between and within species crosses. We discovered that there was no interaction between sex and salt treatment between long-term evolutionary species of mixed Caenorhabditis and short-term learned salt preference of single strains. However, the low salt treatment was statistically significant in mixed strains of Caenorhabditis, as well as single strains. There was also no interaction between salt treatment and sex from one generation to another. Finally, we also noticed partial reproductive isolation between species crosses than within species crosses. It was expected that the chemotactic index would become more extreme with more generations of selection. However, this was not the case. The result goes against the conclusion that long-term adaptation occurs.

Introduction

Through pioneer studies by Sydney Brenner (1974), *Caenorhabditis elegans* (Maupas, 1900), and *C. briggsae* (Dougherty & Nigon, 1949) have become important model systems for studying biological processes. *Caenorhabditis* roundworms have emerged as an excellent experimental model system for studying neuronal and salt mechanisms of chemotaxis. *C. elegans* is a small, transparent, soil-dwelling nematode attracted by compounds that are thought to be associated with its food source, such as bacteria (Ward, 1973). Anatomically, newly hatched *C. elegans* are 0.25 millimeters long and 1 millimeter long when fully mature. Their small size enables them to be observed with a dissecting microscope on petri dishes as they move, eat, mate, develop, and lay eggs. The wild-type *C. elegans* are self-fertilizing hermaphrodites, with rare male individuals

arising in their population at a frequency of <0.2%. The hermaphrodites have XX sex chromosomes, and the males have XO. The males arise by meiotic non-disjunction. However, these males can be propagated by crossing with hermaphrodites and used to carry genetic markers from one hermaphrodite to another. The hermaphrodite is a female whose gonad contains sperm that have already been produced and stored. The mature hermaphrodite can later produce eggs, which are then fertilized by the endogenous sperm. The fertilized eggs undergo some development within the hermaphrodite and are later deposited on agar, where they can hatch to generate small larvae that can develop into adults. Each hermaphrodite can produce between 250 to 300 progenies. Mutant animals are obtained by chemical mutagenesis or exposure to ionizing radiation (Anderson, 1995; Jorgensen and Mango, 2002). Such remarkable characteristics have made *C. elegans* a powerful model for molecular genetic studies in eukaryotic cells. The life cycle of *C. elegans* is relatively short, about 3 days at 22°C from egg to egg-laying adults. The life cycle of *C. elegans* is made up of the embryonic stage, four larval stages (L1-L4), and the adult. The termination of each larval stage is marked with a molt, during which new stage-specific cuticle is synthesized, and the old one is shed off. At this point, there is cessation of pharyngeal pumping, and the animals go into a lethargy (Byerly *et al*., 1976)

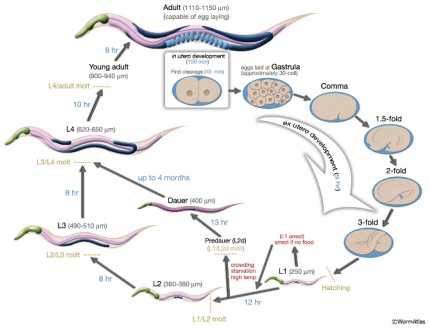

Figure 1: Life cycle of *C. elegans* at 22°C: 0 minute is fertilization. Numbers in blue along the arrows indicate the length of time the animal spends at a certain stage. First cleavage occurs at about 40 minutes post-fertilization. Eggs are laid outside at about 150 minutes post-fertilization and during the gastrula stage. The length of the animal at each stage is marked next to the stage name in µm (wormatlas.org).

Caenorhabditis elegans can be frozen and stored for an indefinite period in liquid nitrogen (Brenner, 1974). *C. elegans* can withstand harsh environmental conditions by switching dauer larval stage that is facultative which enables it to survive about four to eight times than the regular three weeks lifespan (Cassada and Russell, 1975) *Caenorhabditis elegans* is the first multicellular organism to have its whole genome completely sequenced (*C. elegans* Sequencing Consortium 1998), and for this reason, forward and reverse genetics have led to the molecular identification of many key genes in developmental and cell biological processes. About 38% of *C. elegans* protein-coding genes are thought to have orthologs in the human genome (Shaye & Greenwald, 2011), 60% of human genes have an ortholog in the *C. elegans* genome (Kaletter and Hengartner, 2006), and 40% of genes known to be associated with human disease have orthologs in the *C. elegans*

genome (Culetto & Satelle, 2000). Therefore, many discoveries in *C. elegans* can be translated into clinical settings in humans. Other species of *Caenorhabditis*, belonging to the Elegans group, have been discovered in recent years that are closely related to *C. elegans* (Sudhaus & Kiontke, 1996; Kiontke et al., 2002). The Elegans group is a complex of siblings and species that are differentiated by their mode of reproduction, i.e., gonochoristic versus self-fertilizing hermaphrodites. The Elegans group comprises eight species, including *C. brenneri* and

C. remanei (Sudhaus, 1974). The two species, *C. remanei* and *C. brenneri,* are allopatrically distributed, with *C. remanei* located in the temperate regions north of the Tropic of Cancer and *C. brenneri* in the tropics south of the Tropic of Cancer. They are classified as overlapping allospecies since there is no contact or overlap of their geographical ranges. Phylogenetic analysis with molecular data (Chloe *et al.* 2004, Kiontke *et al.* 2004) has shown that the two species are not sister taxa. Previous mating experiments of *C. brenneri* and *C. remanei* have indicated that they are reproductively isolated. They are thus regarded as sibling species since they show almost no differences in morphology, ecology, physiology, and mate recognition (Sudhaus & Kiontke, 2007).

The chemotaxis study of *C. elegans* in the laboratory is conducted by monitoring the migration of worms in gradients of attractants on agar plates (Ward, 1973). Three reasons account for the nervous system of *C. elegans being* relatively easy to study. Firstly, the adult hermaphrodite contains only 302 neurons that are consistently identifiable across animals. Secondly, almost all the automatically defined synaptic connections in adult hermaphrodites have been reconstructed from electron micrographs (Albertson & Thompson, 1976). Thirdly, by using patch-clamp recordings (Goodman et al., 1998) from identified neurons, we can electro-physiologically study the neuronal functions in *C. elegans.* Chemotaxis in *C. elegans* may be regulated by the concentration of attractants detected at a single point on the body (Ward, 1973; Dusenbery, 1980). *C. elegans* possesses pairs of chemosensory organs on its head and tail that are called amphids and phasmids (Ward, 1973). However, the tail is not sensitive to normal chemotaxis (Ward, 1973). It is therefore likely that the worm migrates by detecting the concentration difference between two amphid organs (Ward et al., 1975). *C. elegans* behavior cultured in the laboratory shows a great innate response to food availability, primarily *Escherichia coli* (Baugh & Hu, 2020). Associative learning has been induced in worms by starvation and chemical stimuli, such as sodium chloride (Lim et al., 2018). Under normal conditions, *C. elegans* are attracted to high salt concentrations (Hukema et al., 2006; Saeki et al., 2001). Conversely, worms that are conditioned to grow on salt medium coupled with starvation or food deprivation will learn to avoid higher salt concentrations as a conditioned response (Lim et al., 2018; Saeki et al., 2001). This change in response from attraction to avoidance serves as a behavioral readout for associative learning.

The primary objective of this study was to investigate the long-term adaptation of C. elegans strains and their related gonochoristic species, *C. remanei* and *C. brenneri*, to different salt concentration treatments. We also aimed to characterize the salt preferences of the offspring within and between species crosses. We further aimed to test reproductive isolation between lines that show differences in salt preference. Lastly, we aimed to identify male and female differences.

Materials and Methods:

Worm line origin and maintenance: *Caenorhabditis elegans* strains were obtained from the labs of Patrick Laurent (ULB, Belgium) and Lisebet Temmerman (KU Leuven, Belgium), while strains of *C. remanei* and *C. brenneri* were obtained from the lab of Marie-Anne Felix (Université Paris, France). The strains of *C. elegans* species strains used for this research were ED30117(eE), JT11398(eH), and N2(eN). The *C. remanei* strains were JU1084(rC), JU1086(rB), JU2179(rD), JU2558(rE), JU2744(rH), JU289(rK), JU3277(rM), TMR1001(rA), JU3419(rN). The strains of C. brenneri used were JU1324(bR), JU1398(bW), and JU1815(bX). Upon reception, animals of each strain were aseptically placed on freshly prepared nematode growth medium (NGM) petri plates (Brenner, 1974) that had been previously spread with *Escherichia coli* OP50 as a food source and allowed to grow for 24 h. All cell lines of individual strains were maintained at 18°C, 50% humidity, and a 12 h light-dark cycle.

Proteinase k: It is a broad-spectrum serine protease enzyme. Proteinase K degrades proteins such as DNases and histones, which are associated with DNA. This enables the breaking down of the worm's cuticle, digesting extracellular proteins, and inactivating nucleases, which could degrade genomic DNA (Stiernagle, T. 2006).

Go Taq polymerase: It is a thermostable DNA polymerase derived from Thermus aquaticus, produced by optimized Promega. It was used in PCR to amplify specific DNA sequences of the F1 hybrids

Gel red: Fluorescent dye that was used to stain DNA in agarose gels, allowing the visualization of DNA bands under UV light.

Primers: They were used in PCR to amplify the genomic DNA of interest. To discriminate between closely related Caenorhabditis species (especially the hermaphroditic species *C. elegans* and C. briggsae), one can perform a PCR using species-specific oligonucleotides (Barrière and Félix, 2005). Below are the forward(F) and reverse (R) primer sequences of the Caenorhabditis species used and their corresponding sizes.

C. elegans: CCGCTTGGATTCTATGGATTG and CTCTCCTTGCTCCGGGATTG

C. briggsae: GAACCTGCGAGTGCATGTAC and CCGTCTGCAAACGAACGGGC

C. remanei: CAACGGAGGTATCTGCTCAG and CCGCCGTCAAATTTGCATTC

Primer reference https://www.sciencedirect.com/science/article/pii/S096098220500655X

***E. coli* OP50:** A uracil-auxotrophic strain of *Escherichia coli* used as a food source for Caenorhabditis

DNA ladder: DNA fragments of known size that were used as molecular weight markers in agarose gel electrophoresis. We used a ladder of 100 base pairs.

TBE buffer (10X): It is a Tris-Borate-EDTA commonly used in PCR and electrophoresis. It PCR reaction mixture at an optimal stable pH for DNA polymerase activity during thermal cycling. It provides ions for electrical conductivity so that DNA migrates properly through the gel. We used 10x TBE buffer composed of 216 g Trizma base, 110 g Boric acid, 18.6 g Na_2EDTA, and water.

The prepared 1x TBE buffer from the stock was made by mixing 100 mL of 10X TBE with 900 mL of water.

Cross between *C. remanei* and *C. brenneri* species:

The first stage of this study began by crossing six strains of *C. remanei* males with *C. brenneri* females. To achieve this, we utilized well-maintained nematode strains grown in 1.7% agar nematode growth medium (NGM) seeded with *Escherichia coli* strain OP50 (Brenner, 1974). We used a worm picker to selectively pick the worms under a light microscope near a Bunsen flame. We crossed three males of each *C. remanei* strain with one virgin female of each *C. brenneri* strain in a new plate containing nematode growth medium enriched with fresh bacterial food source. The resulting plates were then placed in an incubator and maintained at 18°C for five days to allow mating to occur.

Below is a table summarizing the crosses performed between the strains. On day five post-mating, we viewed each plate under the light microscope and observed that many offspring were present. We suspected that the progeny were hybrids, which we referred to as F_1. To confirm this hypothesis, we conducted a PCR protocol using species-specific primers, followed by gel electrophoresis.

Table 1: Summary of the cross between different strains of *C. remanei* and *C. brenneri*

C. remanei	rD	rB	rA	rH	rK	rN	rA
C. brenneri	bW	bX	bR	bW	bR	bX	bR
F_1 hybrid	$F_1$1	$F_1$2	$F_1$3	$F_1$4	$F_1$5	$F_1$6	$F_1$3

DNA extraction

The F1 DNA extraction was performed by randomly selecting ten adult worms from each plate and transferring them into separate PCR tubes containing 10 µL of 1X PCR buffer and 1 µL of proteinase K solution. The tubes were labeled $F_1$1 to $F_1$8. $F_1$8 was the control and contained only 10 µL of 1X PCR buffer and 1 µL of proteinase K solution without worms. The 7th PCR tube was a duplicate of $F_1$3 hybrids. The 7th PCR tube was a duplicate of $F_1$3 hybrids. The resulting plates containing the DNA extracts were then placed in a deep freezer maintained at - 70°C for 48 hours for the worms to break up. After 48 hours, the tubes were taken out of the freezer and spun for 5 seconds, then placed into the thermocycler programmed at 65°C for 1 hour and 95°C for 10

minutes. The resulting tubes contain DNA that can be used for PCR and were placed in a freezer maintained at -20°C for 24 hours.

PCR Protocol

The next step involved preparing a master mix composed of 10X PCR buffer, *C. remanei* forward primer (CRE_F), *remanei* reverse primer (CRE_R), *C. brenneri* forward primer (CBR_F), *C. brenneri* reverse primer (CBR_R), deoxyribonucleotide triphosphates (—dNTPS), Taq polymerase, and water.

Table 2A: Master mix preparation

Materials	X/µl	X8/ul
1X buffer	2	16
CRE_F	1	8
CRE_R	1	8
CBR_F	1	8
CBR_R	1	8
dNTPS	0.4	3.2
H_2O	11.4	91.2
Taq	0.2	1.6

The master mix was vortexed for 5 seconds and then spun for an additional 5 seconds. The next step involved pipetting 18 µL each of the master mix solutions and transferring it into eight new PRC tubes and then adding 2 µL each of the previously extracted DNA while maintaining the labels. The next step involved pipetting 18 µL each of the master mix solutions into eight new PRC tubes and then adding 2 µL each of the previously extracted DNA, while maintaining the labels. The tubes were then spun for 5 seconds and placed into the thermocycler under the following conditions.

Table 2B: Thermocycler PCR protocol for Go Taq Polymerase.

Step	Temperature	Time	Cycles
Initial denaturation	95°C	2 minutes	1
Denaturation	95°C	30 seconds	
Annealing	60°C	30 seconds	35
Extension	72°C	1 minute	
Final extension	72°C	10 minutes	
Hold	12°C	∞	

The PCR protocol was run for 2.5 hours, after which the tubes were removed and agarose gel electrophoresis performed.

Agarose gel electrophoresis using GelRed:

Agarose gel electrophoresis was performed by weighing 2 grams of agarose powder and transferring it to a conical flask. The agarose was mixed with 100 mL of 1X TBE buffer and then

heated in a microwave for 1 minute, until the agarose had completely dissolved. The resulting mixture was then cooled to approximately 55°C. The gel was poured into a casting tray with a well-fitted comb and allowed to solidify at room temperature for 40 minutes. The comb was then removed, and the solidified gel was carefully placed in the electrophoresis tank containing 1X TBE running buffer. DNA samples from each PRC tube were then mixed with the loading dye containing GelRed on paraffin paper in the ratio of 1:3 (i.e., 1 µL of dye + 3 µL of DNA sample). We then proceeded to load the gel by first loading the first well with 5 µL of DNA ladder, which contained fragments of 100 bp stained with fluorescent GelRed, and then subsequently loading the remaining wells, each with the corresponding DNA sample, while avoiding air bubbles are cross-contamination between the wells. We finally switched on the power supply and ran the gel at 200V for 45 minutes. After the dye front had migrated for about 70% of the gel length, the power supply was switched off. The gel was then safely removed from the electrophoretic tank using a sterile glove and visualized under UV light. The picture of the gel was then taken and recorded for further analysis, as seen in Figure 2.

Preparation and *E. coli* seeding of NGM Petri plates:

NGM was prepared by mixing 0.3 w% NaCl, 1.7 w%, 400 mL H_2O in an Erlenmeyer flask. The mouth of the flask was covered with aluminum foil and autoclaved at 55°C for 2 h, after which it was cooled in a water bath for 15 minutes. To the flask, 400 µL 1M, 400 µL cholesterol (5mg/mL dissolved in ethanol), 400 µL 1M $MgSO_4$, and 10 mL K_2PO_4 and homogenized by swirling. The NGM was poured onto Petri plates under sterile conditions. Each plate was filled with 2/3 full of agar and then allowed to sit for 30 minutes. To each plate was applied approximately 50 µL of *E. coli* OP50 liquid culture as a drop using a pipette. Occasionally, drops were spread using a glass rod that creates a larger lawn, aiding in worm visualization under the microscope. The *E. coli* OP50 was allowed to grow overnight at room temperature. The seeded plates were packaged in a bacteria transport container and carefully placed in an incubator maintained at 18°C.

A mixture of *C. elegans* strains was made by using a sterilized spatula to chunk pieces of the individual strains and transferring them into a large Petri plate containing NGM seeded with *E. coli* OP50. Similarly, a mixture of *C. remanei* strains was made by chunking nine strains of the *C. remanei* species and transferring them into a large Petri dish containing NGM seeded with *E. coli.* Lastly, an F1 hybrid mix was obtained by chunking six hybrids previously confirmed by gel electrophoresis and transferred into a large plate containing NGM seeded with *E. coli.* The bottom of each plate was labelled with the species name and date. The three plates were placed in an incubator maintained at 18°C for three days, and the worms were then transferred by washing off with M9 into new plates so that as many worms as possible could contribute to the next generation, and genetic diversity could be maintained over time. The plate containing *C. elegans* mix was washed with 3 mL of M9 solution (mixture of 5.8 g anhydrous Na_2HPO_4, 3 g anhydrous KH_2PO_4, 0.5 g NaCl, 1 g NH_4Cl in ddH_2O and autoclaved), 1 mL each was transferred into a new plate containing NGM of high salt concentration, normal salt concentration NGM, and low salt concentration NGM, all seeded with *E. coli.* The same procedures were performed for plates

containing *C. remanei* mix and F1 hybrids. We now had a total of nine plates, each with three different concentrations, for each animal species. The worms were transferred every four days into new NGM plates seeded with bacteria of the corresponding salt concentrations and maintained for up to six generations of evolution under different conditions.

A synchronous population of worms may be needed in an experiment to eliminate variation in results caused by age differences. It consists of treating worms with bleach for a few minutes. The eggs are resistant to bleach, and after several rounds of washing with M9 solution or egg buffer can be transferred onto new NGM plates seeded with bacteria, where they hatch to generate a new synchronous population. Worm synchronization which we term as L1 was performed by washing off plates with 3 mL of M9 using a P1000 pipette and transferring 2 mL into a 15 mL Falcon tube, then transferring the remaining 1 mL of the worm suspension into a new NGM plate of the same salt concentration as the preceding plate from which it was obtained to maintain the cell line. The same procedure was performed for all nine plates. Each falcon tube was filled with 10 mL of distilled water and centrifuged at 500 x RCF for 3 minutes to pellet the worms/eggs. The supernatant was discarded from each tube using a P1000 pipette, leaving the white worm pellet. We immediately added 3 mL of hardcore bleach (1.25 mL 10 M NaOH, 5 mL NaClO (6-14% active chlorine (Merck))) and capped the tubes tightly, then shook several times for 6 minutes. This ensured the effective killing of the worms. At 6 minutes post-bleach incubation we immediately added 10 mL of solution to each tube to stop the bleaching process, then spun the tubes at 500 x RCF for 3 minutes. We immediately discarded the bleach/M9 supernatant, leaving behind the eggs. The eggs were washed further by adding 10 mL of M9 to each tube and spun at 500 X RCF for another 3 minutes. The M9 supernatant was removed and discarded, and the eggs resuspended in 1 mL of M9 buffer. We finally pipetted 1 mL each of the egg suspension and transferred it into new petri plates containing NGM of normal salt concentration, seeded with *E. coli* OP50. The plates were initially placed in the incubator, running at 18°C and 50% humidity, for 144 hours. This enabled the eggs to hatch into juveniles, which developed into young adult pre-egg-laying worms.

Preparation of salt gradient plates:

Linear chemical gradients (Pierce-Shimomura *et al.*, 1999; Iino & Yoshida, 2009) in agar facilitate the study of navigational behaviors in Caenorhabditis and enable the quantification of their movements along spatial gradients. To a greater extent, linear gradients could be achieved with a stimulus-like temperature (Ryu & Samuel, 2002). In this study, we made use of linear chemical gradients to study worm chemotaxis. We prepared salt gradient plates by consecutively pouring NGM of two different concentrations of NaCl over each other in a large square petri plate of 12x12 cm. We elevated one side of the Petri plate using coin stacks at 7mm high, such that the bottom of one side was levelled with the top of the opposite side. This led to a slope of 4°. We proceeded by filling the plate with 70 mL of a melted agar solution of high NaCl concentration, then waited for 10 minutes for the agar to solidify and create a triangular wedge, as shown in figure 3A. The coins were removed, and the plates were made to lie flat. We finally added 70 mL of melted agar solution of low NaCl concentration and waited for 10 minutes to cool. The gradient plates were kept for

24-48 h before being used for subsequent experiments. This enabled diffusion to vertically equilibrate the soluble chemical, creating a linear concentration of the salt (NaCl), high salt concentration on one side of the plate and low salt concentration on the other side of the plate (Iino & Yoshida, 2009).

Worm chemotaxis towards different concentrations of NaCl was done by carefully applying the synchronized worm suspension along the central line separating the square agar plates into high zone salt and low zone salt. To do this, we drew six straight lines of 2 cm apart at the back of each plate to represent compartments of different salt concentrations (figure 3B). Zones 3 and 4 represented the neutral zone, zone 1 was the extreme high salt compartment (HH), and zone 2 was the high salt compartment (H). Conversely, zone 5 was the low salt compartment (L), and zone 6 extreme low salt compartment (LL). We washed each L1 worm plate with 3 mL of M9 and transferred it into a 15 mL Eppendorf tube. The tubes were spun at 500 x RCF for 3 minutes to ensure nearly all the worms sedimented at the bottom. The supernatant from each tube was discarded, leaving behind only 1 mL of worm pellet. We used a P1000 pipette to transfer 1 mL of the worm pellets and carefully applied them along the central line separating zones 1 and 2. We used tissue paper to eliminate excess water from the plates by capillary action. This enabled fast chemotactic migration of the worms along the salt gradient. The plates were allowed to sit for at least 45 minutes before counting mature worms in different compartments under a Leica stereoscope. Our interest was focused on zones that migrated to the high zones and low zones, but not the neutral zone.

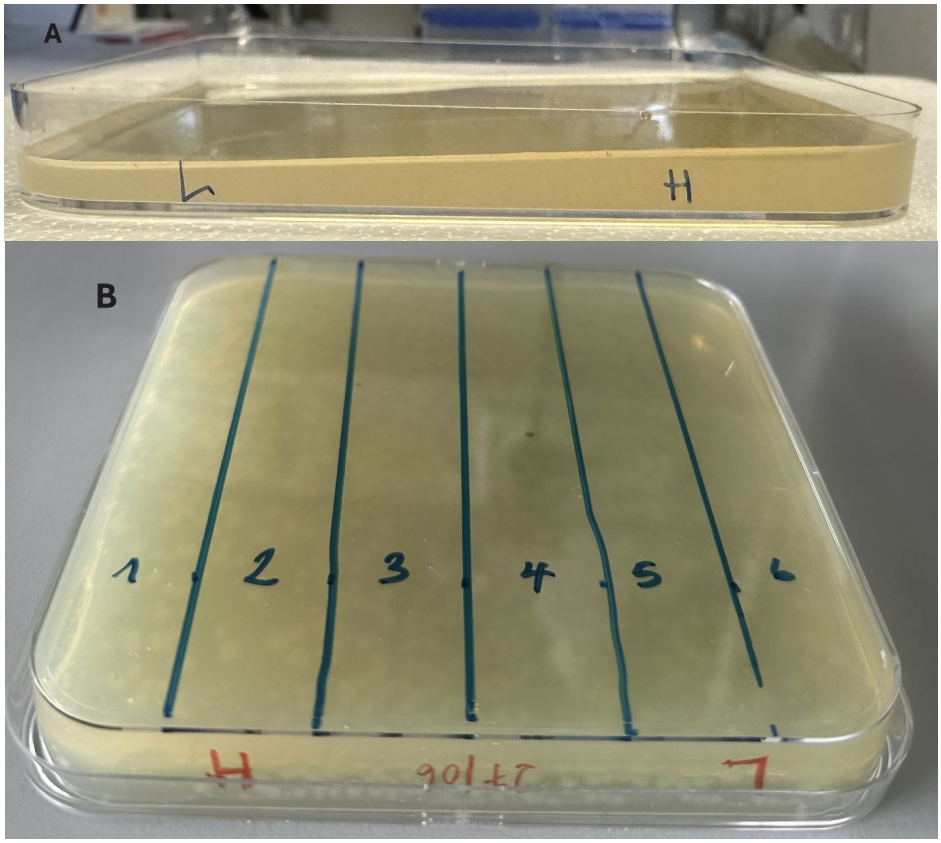

Figure 2. Gradient preparation setup A) Filled plate with high salt-concentrated agar tilted at one end to form a slope and made to solidify. B) Complete plate with high salt agar and low salt agar divided into zones (1-6) from high salt zones to low salt zones.

We determined the **chemotactic index** as in (Kano *et al.*2008), by calculating the difference between the number of worms that migrated to the high salt zones (1 & 2) and the low salt zones (5 & 6), all divided by the total number of worms counted in all four zones. *Caenorhabditis elegans* were counted as hermaphrodite females. *Caenorhabditis remanei* and *Caenorhabditis brenneri* were separately counted as males and females. We kept track of the time interval during which counting was done for each after the worm pellets were applied to the square plate.

For **statistical analysis** and data visualization, we used RStudio version 4.5.0 (R Core Team, 2007) and ANOVA.

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Results

Agarose gel electrophoresis confirming PCR amplification of F1 progeny

We performed PCR and agarose gel electrophoresis of the parental strains *C. elegans*, *C. brenneri*, and *C. remanei* before proceeding with the generation of the hybrids. Figure 3 confirmed the parent strains of Caenorhabditis from which the hybrids were generated.

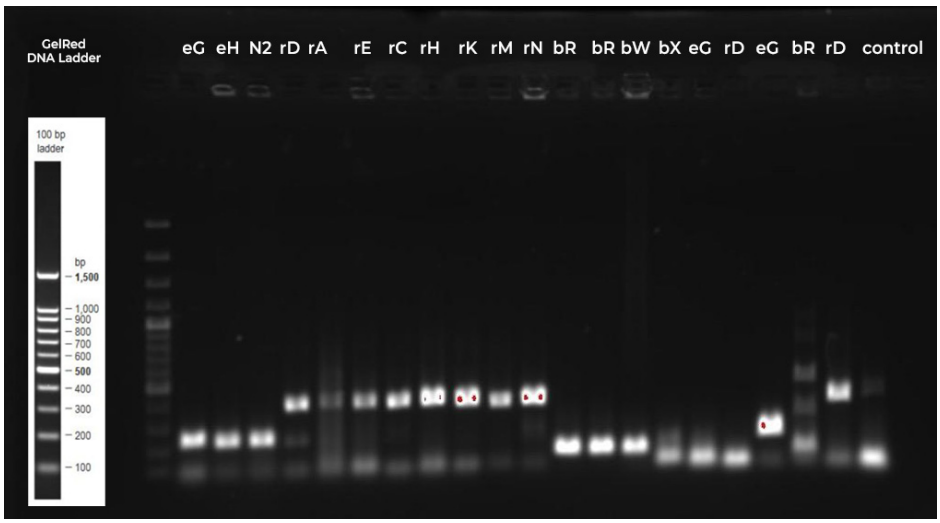

Figure 3. Agarose gel electrophoresis confirming the different parent strains of *C. elegans*, *C. remanei* and *C. brenneri* used for crossing during this project. The strains were electrophoresed alongside the GelRed at the beginning, and a control sample containing only water. The names of the different strains used for each species are: *C. elegans*: ED30117[eE], JT11398[eH], and N2[eN)]; *C. remanei*: JU1084[rC], JU1086[rB], JU2179[rD], JU2558[rE], JU2744[rH], JU289[rK], JU3277[rM], TMR1001[rA], JU3419[rN]; and *C. brenneri*: JU1324[bR], JU1398[bW], JU1815[bX]. Each parent strain indicated a single band.

PCR products from six F1 offspring ($F_1$1-$F_1$6) derived from crosses between *C. remanei* males (rD, rB, rA, rH, rK, rN) and *C. brenneri* females (bW, bX, bR, bX) were confirmed by agarose gel electrophoresis (Figure 4). The $F_1$2 and $F_1$6 were negative, suggesting that the crosses were not successful or that the females were no longer virgins at the onset of mating.

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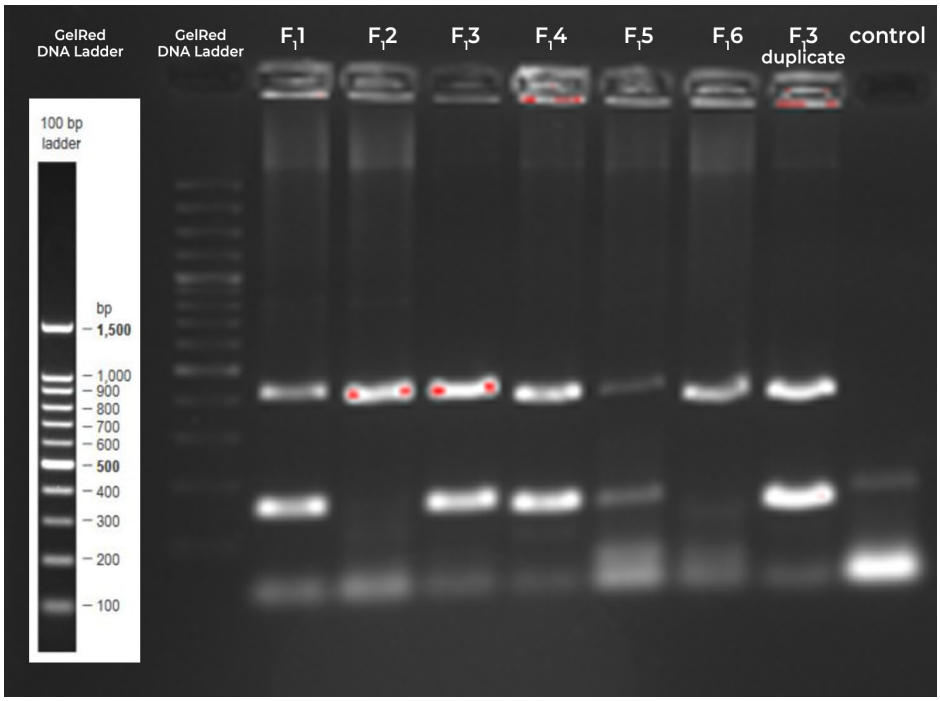

Figure 4. Agarose gel electrophoresis of the F_1 hybrids. The strains were electrophoresed alongside the GelRed at the beginning, and a control sample containing only water. The symbols of the strain used were $F_1$1[rD.bW], F_1`2[rB.bX], $F_1$3[rA.rB], $F_1$4[rH.bW], $F_1$5[rK.bR], $F_1$6[rN.bX], $F_1$3[rA.bR]. Each hybrid is represented with a double band.

Long-term evolutionary adaptation to different salt concentrations.

Caenorhabditis cultured under standard conditions has been shown to exhibit positive chemotaxis towards sodium chloride (NaCl). The animal will exhibit negative chemotaxis upon prolonged exposure to salt under starvation. Such learning and memory retention in Caenorhabditis is plastic. We calculated the mean chemotactic index of worms, which enabled us to determine the level of plasticity regarding learning and memory retention in Caenorhabditis under different salt treatments. We compared our results of the mixed strains evolving under different salt treatments with those of BSc student Luca Pignatti data for single-strain treatments, as he had more data for single-strain treatments than me. The sexes did not differ in salt chemotaxis in all *C. remanei* strains (Fig. 5a and b). Looking at our mixed worm population that evolved under different salt treatments, we found that there was no interaction between sex and salt treatment, nor was sex significant in a model with sex and salt (Fig. 5a and b). We analyzed the data by looking at salt treatment only, and the anova analysis revealed that the effect of treatment for *C. remanei* mix was

significant (F=6.6, p<0.01). There was a difference in salt treatments between the low, normal, and high salt treatments. The low salt treatment had a 0.4 lower index than the normal and high salt treatments (p = 0.02). The high and normal salt treatments were not significantly different (Fig. 5a and b). Finally, we compared our results for the mixed population evolving under different salt conditions with a single *C. remanei* strain for which chemotaxis index data were available (from Luca Pignatti) and found the salt treatment significant (F=4.9, p<0.05). This was driven by low salt treatment, which had an index value 0.592 lower than the high salt treatment and was significant at the 5% level (p-value=0.04)

Table 3A: Summary of statistical coefficients for salt treatments for mixed evolved *C. remanei* and *C. remanei* learned salt preference

	estimate	Std error	t value	Pr(>|t|)
Intercept	-0.005	0.19	-0.025	0.98
Low Salt treatment *C. remanei*	-0.591	0.27	-2.221	0.04*

The experimental population of mixed *C. elegans* strains (Fig.5e), which underwent high salt treatment, showed a significant difference in treatment response (F=4.3, p-value=0.03). The mean index value was 0.31 greater than the normal and low salt treatments. It was significant at the 5% level (p-value = 0.033).

Table 3B: Summary for statistical coefficients of salt preference for *C. elegans*

	Estimate	Std. Error	t value	Pr(>|t|)
Intercept	-0.04	0.001	-0.436	0.669
Salt treatment *C. elegans* mix High	0.316	0.136	2.333	0.033 *
Salt treatment *C. elegans* mix Low	-0.05	0.141	-0.332	0.744

There was also no significant difference between the *C. elegans* (Fig.5e) mixed population evolving under different salt treatments, compared with the short-term learned memory effect of a single strain (from Luca Pignatti), as observed in the summary table below.

Table 3C: Summary table for statistical coefficients of salt treatments for *C. elegans*

	Estimate	Std. Error	t value	Pr(>|t|)
Intercept	-0.04	0.148	-0.292	0.77
C. elegans mix High	0.316	0.202	1.566	0.13
C. elegans mix Low	-0.047	0.210	-0.223	0.83
C. elegans High	0.362	0.191	1.893	0.07

There was no significant difference in sex and interaction within salt treatment in the experimental mixed population of the F1 hybrid (*C. remanei* x *C. brenneri*) evolving in different salt concentrations (Fig. 5c and d). When analyzed by sex and salt treatment without interaction, we observed a significant difference (F=3.7, p-value=0.04). This was driven by high and low salt treatments. The high salt and low salt treatments had index values 0.5 and 0.4 higher than the intercept (0.4). They were both significant at the 5% level (p-values 0.01, 0.05). The male sax was not significant, as observed in the summary table below for the coefficients.

Table 3D: Summary table for statistical coefficients of evolution treatment and sex for F1 hybrids

	Estimate	Std. Error	t value	Pr(>|t|)
Intercept	-0.398	0.156	-2.543	0.02 *
Salt treatment F1mix High	0.472	0.179	2.633	0.01 *
Salt treatment F1mix Low	0.387	0.186	2.073	0.05 *
Male sex	0.081	0.144	0.562	0.58

Finally, we analyzed the results based on the evolution treatment only, and all the different salt treatments were significant (F=3.8, p-value 0.04). The normal, low, and high salt treatments were all different (Fig. 5c and d). The high and low salt treatments were 0.47 and 0.39 higher than the normal salt (0.36), significant at 5% levels, respectively (p-values 0.01, 0.05). The summary coefficient table is shown below.

Table 3E: Summary of statistical coefficients for species for F1 hybrids

	Estimate	Std. Error	t value	Pr(>|t|)
Intercept	-0.358	0.1372	-2.605	0.01 *
Salt treatment F1mix High	0.473	0.1772	2.667	0.01 *
Salt treatment F1mix Low	0.387	0.1841	2.099	0.05 *

The short-term learned response of the *C. remanei* strain (from Luca Pignatti) in low salt treatment was significant (F=3.9, p-value 0.04). It had an index value of 0.93, lower than the high salt treatment (0.33). It was significant at 5% level (p-value 0.02). The learned response of the *C. remanei* strain in low salt treatment, *C. brenneri* strains in low salt and high salt treatments was all the same (Fig. 5c and d), as shown in the table below.

Table 3F: Summary table of statistical coefficients for evolution treatment of *C. remanei* strains

	Estimate	Std. Error	t value	Pr(>|t|)
Intercept	0.333	0.318	1.048	0.30
Salt treatment *C. brenneri* Low	-0.445	0.405	-1.100	0.28

Salt treatment *C. brenneri* High	-0.338	0.374	-0.904	0.37
Low Salt treatment	-0.931	0.370	-2.512	0.02 *

There was no significant difference in evolution treatments of the mixed population evolving in low salt and high salt treatments when compared to the short-term learn response of the *C. remanei* strains (from Luca Pignatti) in low salt and high salt treatments (Fig. 5c and d). However, only the *C. remanei* strain in low salt treatment was significant (F=2.7, p-value 0.03). It had a mean index value 0.93 lower than the high salt treatment of *C. brenneri* (0.33) at less than 5% level (p-value 0.001) as seen in the coefficient summary table below.

Table 3G: Summary of statistical coefficients of evolution treatments for mixed population and *C. remanei* strain

	Estimate	Std. Error	t value	Pr(>|t|)
Intercept	0.333	0.280	1.188	0.240
Salt treatment *C. brenneri* Low	-0.446	0.358	-1.247	0.217
Salt treatment F1mix High	-0.218	0.334	-0.654	0.516
Salt treatment F1mix Low	-0.304	0.344	-0.886	0.380
Salt treatment *C. remanei* High	-0.338	0.330	-1.025	0.310
Salt treatment *C. remanei* Low	-0.931	0.327	-2.847	0.001 **

Finally, we analyzed the results by salt preference between the mixed evolved population in low and high salt treatments, irrespective of sex, with the learned response population strains of *C. remanei* and *C. brenneri* (Fig. 5c and d). The low salt treatment was significant (F=5.3, p=0.02). The low treatment had an index 0.38 lower than the high salt treatment. It was significant at the 5% level (p-value 0.02) as seen in the coefficient summary table below.

Table 3H: Summary table of statistical coefficients of salt treatments for the mixed evolved population and *C. remanei* and *C. brenneri* strains (from Luca Pignatti).

	Estimate	Std. Error	t value	Pr(>|t|)
Intercept	0.09944	0.118	0.843	0.40
Low Salt	-0.380	0.164	-2.313	0.02 *

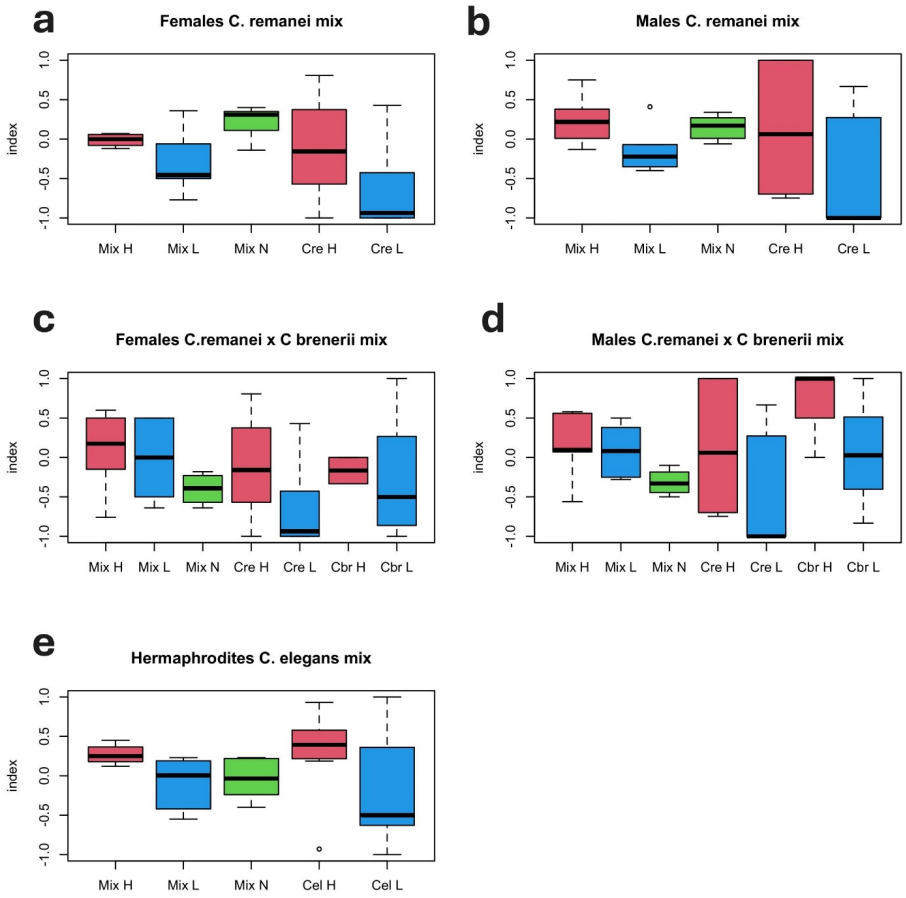

Figure 5. Boxplots for the long-term evolutionary adaptation of mixed Caenorhabditis species and single learned salt preference of single strains (from Luca Pignatti). *C. remanei* [Cre], *C.brenneri* [Cbr], *C.elegans* [Cel], High salt treatment[H], Low salt treatment [L], Normal salt [N]

Chemotactic index by generation.

There was no interaction between the mixed populations of *C.elegans* evolving in different salt concentration treatments from one generation to the next, ie, the slope of the index over time did

not differ between the salt treatments (Fig. 6a). The treatments overall differed as described above,

	Estimate	Std. Error	t value	Pr(>|t|)
Intercept	0.16467	0.21295	0.773	0.45
Generation	-0.059	0.055	-1.087	0.28
Salt treatment *C. elegans* mix High	-0.084	0.300	-0.280	0.78
Salt treatment *C. elegans* mix Low	0.075	0.301	0.250	0.81
Generation-Salt treatment *C. elegans* mix Low	-0.035	0.077	-0.451	0.66
Generation-Salt treatment *C. elegans* mix High	0.111	0.075	1.475	0.16

but the index did not become more extreme as the generations increased.

Table 4A: Summary of statistical coefficients for the evolution treatment of *C. elegans* mixed population

There was no interaction between salt treatment and sex from one generation to the next in the experimental population of mixed *C. remanei* strains evolving in different salt treatments (Fig. 6b). The high, low, and normal treatments were all the same as seen on the table below.

Table 4B. Summary of statistical coefficients of the evolution treatment, and sex for the mixed population of *C. remanei*

Intercept	Estimate	Std. Error	t value	Pr(>|t|)
Generation	0.103	0.296	0.348	0.732
Salt treatment-Male sex *C. remanei* mix Normal	0.023	0.073	0.391	0.700

Salt treatment-Female sex *C. remanei* mix High	0.147	0.419	0.352	0.729
Salt treatment-Male sex *C. remanei* mix High	-0.269	0.503	-0.535	0.559
Salt treatment-Female sex *C. remanei* mix Low	0.299	0.503	0.594	0.559
Salt treatment-Male sex *C. remanei* mix Low	-0.176	0.409	-0.431	0.671
Generation: Male sex & Salt treatment *C. remanei* mix Normal	0.004	0.409	0.011	0.992
Generation: Female sex & Salt treatment *C. remanei* mix High	-0.058	0.103	-0.557	0.584
Generation: Male sex & Salt treatment *C. remanei* mix High	0.009	0.121	0.078	0.939
Generation: Female sex & Salt treatment *C. remanei* mix Low	-0.097	0.103	-0.944	0.356
Generation: Male sex & Salt treatment *C. remanei* mix Low	-0.100	0.103	-0.969	0.344

There was no interaction between salt treatment and sex from one generation to the next in the experimental population of mixed F1 hybrids (*C. remanei* x *C. brenneri*) species evolving in different salt treatments (Fig. 6c). However, there was significant interaction between treatment and female sex in the mixed F1 population that evolved in low salt treatment. It was 1.28 higher than the intercept (0.462) and significant at 5% level (p-value 0.04). The summary table for the coefficients is outlined below.

Table 4C: Summary of statistical coefficients for sex and salt treatments of mixed (*C. remanei* x *C. brenneri*).

	Estimate	Std. Error	t value	Pr(>|t|)
Intercept	-0.462	0.388	-1.191	0.25
Generation	0.019	0.103	0.185	0.86
Normal Salt treatment-Male sex F1mix	-0.079	0.548	-0.144	0.89
High Salt treatment -Female sexF1mix	0.510	0.534	0.955	0.35
High Salt treatment -Male sex F1mix	0.475	0.554	1.401	0.18
Low Salt treatment Female sexF1mix	1.283	0.577	2.224	0.04 *
Low Salt treatment – Male sexF1mix	1.073	0.546	1.964	0.07
Generation-Normal Salt treatment -sexF1mix	0.051	0.145	0.348	0.73
Generation-High salt treatment-Female sexF1mix	-0.007	0.139	-0.050	0.96
Generation-High salt treatment Male sexF1mix	-0.061	0.139	-0.436	0.67
Generation-Low salt treatment Female sex F1mix	-0.242	0.145	-1.672	0.11
Generation-Low salt treatment Male sexF1mix	-0.165	0.140	-1.178	0.25

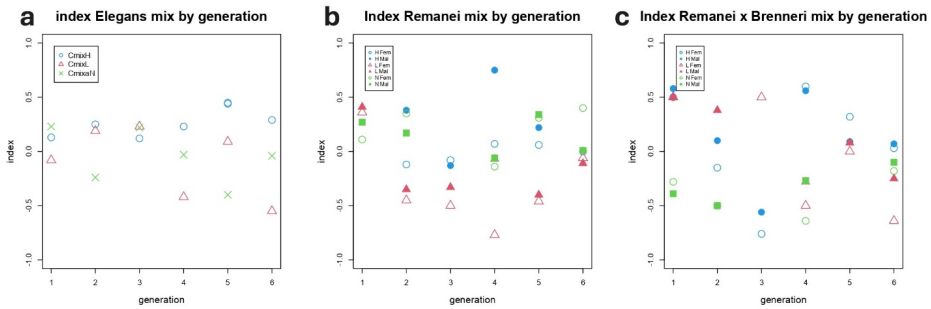

Figure 6: chemotactic index by generation for *C. elegans* mix, *C. remanei* mix and *C. remanei* x C. *brenneri* mix

Reproductive output within and between species crosses.

The mean index value of the cross within species was 7.90 greater than that of the cross between species (16.25), as expected if adaptation to different salt conditions results in reproductive isolation; however, the difference was not significant (Fig. 7). The summary table is outlined below.

Table 5: Summary of statistical coefficient between and within species cross.

	Estimate	Std. Error	t value	Pr(>|t|)
Intercept	16.25	3.283	4.950	4.19e-06 ***
type2within	7.90	4.642	1.702	0.0928

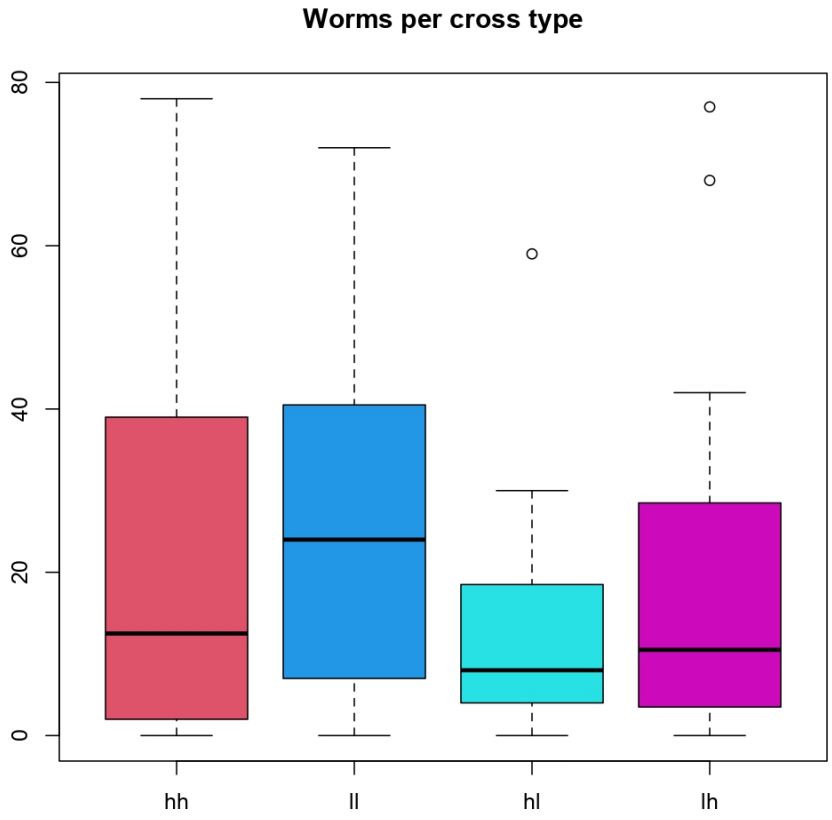

Figure 7: Boxplots for species cross within and between salt treatments; high[h], low[l]

Discussion

It has previously been demonstrated that salt chemotaxis in Caenorhabditis is plastic and is influenced by sex, species, and the former salt milieu in which Caenorhabditis was cultured. In this study, we tried to investigate the interaction between sex and salt-evolution treatment. Our results showed that sex was not influenced by salt treatment in the long-term evolved mixed population of e *C. remanei* as well as short-term learned single strains. There was no interaction between sex and salt treatment between the males and females of the *C. remanei* mixed population. However, equally noticed that salt concentrations had an impact on worm migration of both the

mixed population and the single strains of *C. remanei*. This is evidence that long-term selection can affect the plastic response of worms. Surprisingly, the low salt and normal salt did not behave as expected. This could be caused by excessive bacterial contamination in some gradients that may have influenced the worm's preference. It is also possible that there was not enough statistical power to detect the difference that could be detected with one replicate.

The mixed population of long-term evolved *C. elegans* was attracted more towards the high salt environment than the low and normal salt environments. However, the low salt and normal salt treatments did not behave as expected. This could also be caused by excessive bacterial contamination in some gradients that may have influenced the worm's preference. It is also possible that there was not enough statistical power to detect the difference that could be detected with one replicate

Our results also showed that there was no interaction between sex within mixed populations of the hybrids and single strains. However, we noticed a difference in sex and salt treatments between the mixed populations of hybrids. The high salt treatment had a positive index as expected, and surprisingly, the low salt treatment also had a positive index, which was unexpected. Low salt treatment of the mixed long-term evolved population of hybrids and single strains of short-term learned salt preference was significant and behaved as expected.

Our results equally showed that there was no interaction between sex and salt treatments from one generation line to the next. We expected the low salt treatment to have a constant negative slope, the high salt treatment to have a constant positive slope, and the normal salt treatment to have an overall zero slope, but that was not the case. The index for the different salt treatments kept fluctuating from one generation to the next. This could be caused by excessive bacterial contamination in some gradients that may have influenced the worm's preference. It is also possible that there was not enough statistical power to detect the difference that could be detected with one replicate.

There was an overall lower reproductive output in species crosses, but we witnessed a higher reproductive success rate within species crosses than between species crosses. This was caused by partial reproductive isolation in the species. Low experimental sample size and limited generation time could account for this observation.

Finally, it was expected that the chemotactic index would become more extreme with more generations of selection. However, this was not the case. The result goes against the conclusion that long-term adaptation occurs. Again, as seen in Figure 6a, the lack of significance is probably due to the experiment not working well for a particular week.

Conclusion

Salt chemotaxis in Caenorhabditis is plastic and influenced by different salt concentrations. More experimental studies to investigate the interaction between sex and salt treatment need to be further carried out using larger samples and a prolonged generation time.

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