# Temperature effects on hybrid reproductive isolation between *C. brenneri* and *C. remanei*

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| ***Keywords*** | ***Abstract*** |
|---|---|
| Caenorhabditis<br>Reproductive isolation<br>Hybrid incompatibility<br>Temperature stress<br>Haldane's rule | Reproductive isolation plays an important role in maintaining species boundaries. The strength of this isolation can vary depending on genetic and environmental conditions.This study investigated postzygotic reproductive isolation between *Caenorhabditis brenneri and Caenorhabditis remanei*, two closely related nematode species previously described as reproductively isolated. Although crosses succeeded in the lab. Interspecific and intraspecific crosses were performed at 18°C and 24°C to test whether temperature affects egg production, $F_1$ offspring production, offspring sex ratio, and hybrid fertility. Crosses were established by placing one virgin female with three males. The number of eggs, number of $F_1$ offspring, and offspring sex were then recorded. Egg production did not differ significantly between temperatures or cross types, suggesting that reproductive isolation does not involve fecundity. In contrast, temperature had a significant effect on total $F_1$ offspring production, with more offspring at 18°C than at 24°C. Fertility tests indicated that $F_1$ hybrid females remained fertile, whereas $F_1$ hybrid males were sterile when backcrossed with both parental species, consistent with Haldane's rule. Sex ratio did not differ significantly between both temperatures.Overall, these results suggest that *C. brenneri* and *C. remanei* show strong but incomplete reproductive isolation, with temperature mainly affecting postzygotic offspring viability rather than egg production or sex ratio. |

## Introduction

The genus *Caenorhabditis* is widely used in biological studies because of its short life cycle and ability to produce large numbers of offspring within a short period of time (Brenner , 1974). Species of this nematode genus are distributed worldwide and often show only subtle morphological differences, making species identification challenging (Sudhaus & Kiontke, 2007). Species relationships can be assessed using phylogenetic analyses, while reproductive compatibility is often tested through mating assays. This study investigates the strength of postzygotic reproductive isolation between *Caenorhabditis remanei* and *Caenorhabditis brenneri*. Previous work has described these species as reproductively isolated (Sudhaus & Kiontke, 2007). However, recent laboratory crosses suggest that the extent and nature of this reproductive isolation should be re-evaluated(Zouberou P., MSc thesis). Because the published study did not specify the temperature at which the crosses were maintained, the present study tested crosses at two different temperatures. This allowed us to assess whether temperature influences hybrid production and viability, and whether temperature differences could partly explain discrepancies between the present results and previous reports.

Postzygotic reproductive isolation refers to barriers that occur after mating and fertilization, when hybrids either fail to develop, show abnormal development, or are sterile (Bi *et al.*, 2015). *C. remanei* and *C. brenneri* are allopatrically distributed, with *C. remanei* found mainly in northern temperate regions and *C. brenneri* in circumtropical regions (Sudhaus & Kiontke, 2007). These species are thought to have been geographically isolated for approximately 20 million years, preventing gene flow and allowing genetic differences to accumulate (Memar *et al*., 2019; Sudhaus & Kiontke, 2007). They show asymmetrical reproductive isolation: offspring are produced only when *C. brenneri* females are crossed with *C. remanei* males, but the resulting male hybrids show severe inviability (Sudhaus & Kiontke, 2007).

Because this experiment focuses on hybridization, it is relevant to consider Haldane's rule. Haldane's rule states that when hybrids between two species show sterility or inviability, the affected sex is usually the heterogametic sex, which in many *Caenorhabditis* species is the male (Orr, 1997). This pattern has been widely supported across many animal groups with heterogametic males (Schilthuizen *et al*., 2011). Haldane's rule cannot be explained exclusively by characteristics of male biology, such as stronger sexual selection: In species where the females are the heterogametic sex such as butterflies, crosses shows that female hybrids are sterile while males are normally fertile, an example of it is the crosses between two lepidoptera species. The female hybrids of *Heliconius melpomene* and *H. cydno* are completely sterile while males do not undergo any significant changes (Naisbit *et al*., 2002). Evidence has been observed for rarity and sterility of hybrid males in crosses of three *Silene dioecious* plant species with heteromorphic (XY) sex chromosomes (Brothers & Delph, 2010).

One major explanation for Haldane's rule is the dominance theory. According to this theory, many genetic incompatibilities causing hybrid sterility or unviability are recessive. In the heterogametic sex, recessive incompatibilities on the X chromosome are fully expressed because there is no second copy of the X chromosome to compensate for them (Turelli & Orr, 1995).

Hybrid incompatibility has already been studied in nematodes related to *C. elegans*, such as *C. nigoni* and *C. briggsae*. In these species, hybrid incompatibility loci and male-inviability loci were frequently found on the X chromosome (Bi *et al*., 2015). This was shown by introgressing fragments of the *C. briggsae* X chromosome and autosomes into the *C. nigoni* genetic background and assessing their effects on viability (Bi *et al*., 2015). These results support the large X effect, which proposes that the X chromosome plays a disproportionate role in hybrid incompatibility and unviability. Similar patterns of large X effects and male sterility have also been tested in crosses between *C. remanei* and *C. latens*, suggesting that comparable mechanisms may occur in crosses between *C. brenneri* and *C. remanei* (Bundus *et al.*, 2018).

The offspring obtained from interspecific crosses will be assessed for viability and fertility as indicators of hybrid incompatibility. This approach will allow the presence and fitness of hybrids to be evaluated phenotypically, without requiring DNA extraction. In addition, temperature will be considered an important factor affecting development in *Caenorhabditis*. Developmental rate generally increases at higher temperatures, reducing the time required to reach maturity. This effect has been well studied in *C. elegans* and may also be relevant in related species (Mata-Cabana et al., 2022; Sikkink et al., 2014). *C. remanei* shows strong temperature-dependent effects on offspring survival rates and can respond to high temperatures, such as 24°C, through plastic changes in gene expression that increase heat resistance (Lind *et al*., 2016). However, less is known about comparable heat resistance in *C. brenneri*. Therefore, comparing development and viability under different temperatures may help clarify how temperature influences hybrid fitness in offspring *C. remanei* and *C. brenneri*. Only a few studies have demonstrated temperature effects on hybrid fitness. An example of it can be seen in the parasitic wasp genus *Nasonia*, temperature stress increased hybrid dysfunction by enhancing mortality and sterility, and by revealing cryptic incompatibilities between parental genomes that were not detectable under standard temperatures (Koevoets et al., 2011). These findings suggest that temperature can influence the expression of Dobzhansky–Muller incompatibilities and support the hypothesis that heat stress may similarly intensify or expose hybrid incompatibilities in other taxa, such as nematodes (Koevoets et al., 2011). The same hypothesis was confirmed in hybrids of the flour beetles *Tribolium castaneum* and *T. freeman*. Hybrids raised at hightemperatures expressed more morphological abnormalities (Wade *et al*., 1999).

## Methods

16 strains (Appendix 1) of *C. brenneri* and *C. remanei* provided by Marie Ann Felix were cultivated on nematode growth medium (NGM) plates and observed alive using bright-field microscopy. Mating tests were conducted by transferring one virgin *C. brenneri* female and three *C. remanei* males onto a fresh NGM plate using a worm picker. Plates were incubated at 18°C or 24°C, in chambers with a 12-hour light dark cycle, and the number of eggs produced was counted after 24 hours. Two to three days after the transfer, the offspring were counted and sexed. Nematode sex was determined based on tail morphology and the presence of eggs or a vulva (Sudhaus & Kiontke, 2007). Female tails are slender and pointy, like those of *C. elegans* hermaphrodites. Male tails are broader and can sometimes be mistaken for the head.

Crosses were performed multiple times using all possible strain combinations for intra-specific crosses. Inter-specific crosses were also performed as controls. The *C. remanei* and *C. brenneri* strains used in this experiment originated from different locations. Each strain combination was tested at least 2-3 times to evaluate variability in cross success between different strains, and determine which crosses are most interesting to follow up in further research.

Crosses were carried out at two temperatures. First, crosses were performed at 18°C, a temperature at which preliminary data suggested that viable hybrids could be produced (Zouberou P., MSc thesis). Secondly, crosses were performed at 24°C, which is close to the upper tolerable temperature range of the species, to determine whether increased temperature negatively impacts hybrid production and viability (Lind *et al.*, 2016).

Because the parental species cannot be morphologically distinguished, it was necessary to confirm whether the observed offspring were indeed hybrids. This could be tested by assessing the fertility of the $F_1$ males, since only hybrid males would be infertile. We therefore crossed $F_1$ males and females with individuals of the opposite sex from both parental species. If $F_1$ males failed to produce offspring, while $F_1$ females remained fertile, this was consistent with Haldane's rule and supported the hybrid status of the offspring.

A second and stronger confirmation method involves DNA extraction from the hybrids and the parental strains, followed by PCR amplification using species-specific primers and gel electrophoresis. This method can differentiate interspecific hybrids, which will have bands from both parental species amplified, from offspring of mistakenly used non-virgin mothers, which would have already mated within the same species and would produce only the band of one parental species in the same PCR.

DNA extraction of $F_1$ was performed by selecting 10 adult worms of the chosen plate and transferring into a PCR tube containing

20 µL of 1X PCR buffer and 1 µL of proteinase K solution. The PCR tubes were then placed in a freezer at -80°C for 20 minutes to break up the worms. The last step places the tubes in a thermocycler programmed to 65°C for 1 hour and 95°C for 10 minutes.

The next step involves preparing a master mix composed of 10X PCR buffer, *C. remanei* forward primer (CRE_F), *C. 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 3A).Below are the forward(F) and reverse (R) primer sequences used , along with their corresponding sizes (Barrière & Félix, 2005).

*C.brenneri* : CCTTAGTAGTGGCCTCAATGGTGT and CACAACCTATCACTTCACTCTGGC (bp 302)

*C.remanei* : CAACGGAGGTATCTGCTCAG and CCGCCGTCAAATTTGCATTC (bp 391)

| Materials | X/µl per tube |
|---|---|
| 1X buffer | 2 |
| CRE_F | 1 |
| CRE_R | 1 |
| CBR_F | 1 |
| CBR_R | 1 |
| dNTPS | 0.4 |
| Taq polymerase | 11.4 |
| Water | 0.2 |

Table 3A: Master mix preparation

The master mix was vortexed for 5 seconds, then centrifuged for an extra 5 seconds. Next step involved pipetting of 18 µl of the master mix in new PCR tubes, then adding 2µl of the DNA extraction made previously. The tubes were then placed into a thermocycler under the program shown in Table 3B. An agarose gel electrophoresis was then performed.

| 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 minutes | |
| Final extension | 72°C | 10 minutes | |
| Hold | 12°C | ∞ | |

Table 3B: Thermocycler PCR protocol

## Statistical analysis:

The crosses that produced no eggs or adult offspring were divided into two categories. The first category included crosses that failed because one of the parents died or disappeared, possibly due to poor picking technique, contamination, drying of the plate, or worms escaping from the edge of the agar. The second category included crosses that "successfully failed," meaning that the female and the three males were present and appeared healthy after 3 days, but no eggs or offspring were observed. These cases may indicate biological incompatibility or reduced reproductive success rather than a technical failure.

The successful crosses were then analyzed by comparing the number of eggs and $F_1$ offspring produced intraspecific and interspecific crosses at the two treatment temperatures. In addition, the sex ratio of the $F_1$ offspring was compared between temperatures to evaluate whether temperature negatively impacted male production as expected if it strengthens Haldane's rule of male inviability.

The data were analyzed with R studio running R version 4.5.1 to make statistical test models and graphs for interpretation. The number of eggs and $F_1$ data was first tested to see if it was normally distributed using a Shapiro-Wilk test. The results showed a significant deviation from a normal distribution with a $p = 2.613.10^{-9}$ for eggs and $p = 7.521.10^{-16}$ for $F_1$ offspring. A normal Q-Q plot was also plotted for both results, but neither of them showed a normal distribution. As the classic linear model is inappropriate, a Poisson model was attempted, however it showed strong overdispersion for both results. The final statistical model used for further analysis is the negative binomial.

The sex ratio was analyzed using a binomial model due to its proportional nature. The 0 $F_1$ offsprings were excluded because sex ratio is undefined when no offsprings are produced

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# Results

## Strain used

Each possible strain combination for hybrid production was tested 3 times, and offspring production was recorded. Crosses between female *C. remanei* and male *C. brenneri* produced no offspring, as previously found (Sudhaus & Kiontke, 2007). In the reciprocal cross, female *C. brenneri* crossed with male *C. remanei*, satisfactory results were obtained only with the *C. brenneri* strain bW (Appendix 2). Therefore, strain bW was chosen as the main strain for interspecific crosses between *C. brenneri* and *C. remanei*.

## Egg analysis

Egg production across the different crosses is shown in Figure 1A. Overall, egg production in both intraspecific and interspecific crosses was lower than expected for *Caenorhabditis* species. *Caenorhabditis* typically produce large numbers around 150-300 eggs per female in lab conditions (Sudhaus & Kiontke, 2007). However, in the present experiment, most observed eggs counts were closer to approximately 40–60 eggs.

A statistical model was fitted to test whether egg production was affected by temperature, cross type, or their interaction. The interaction between temperature and cross type was not significant, indicating that the effect of temperature on egg production did not differ meaningfully between the different cross types. Therefore, a simpler model including only the main effects of temperature and cross type was used for further interpretation. The model predicted fewer eggs at a higher temperature than at a lower temperature, with an estimated mean of 44.5 eggs at high compared with 59.5 eggs at low temperature. However, the difference was not statistically significant ($\chi^2 = 0.65$, $p = 0.42$; Figure 1B). Even if the trend suggests a possible reduction at higher temperatures, the available data does not provide sufficient evidence to conclude that temperatures affect egg production. Similarly, egg production differed only slightly between cross types. The interspecific cross had a lower predicted number of eggs, with an estimated mean of 46.2 eggs, compared with the intraspecific crosses of *C. brenneri* and *C. remanei*, which had means of 52.0 and 56.8 eggs, respectively. It should be noted that these differences were not statistically significant ($\chi^2 = 0.06$, $p = 0.97$). Therefore, the results do not support a significant effect of cross type on egg production.

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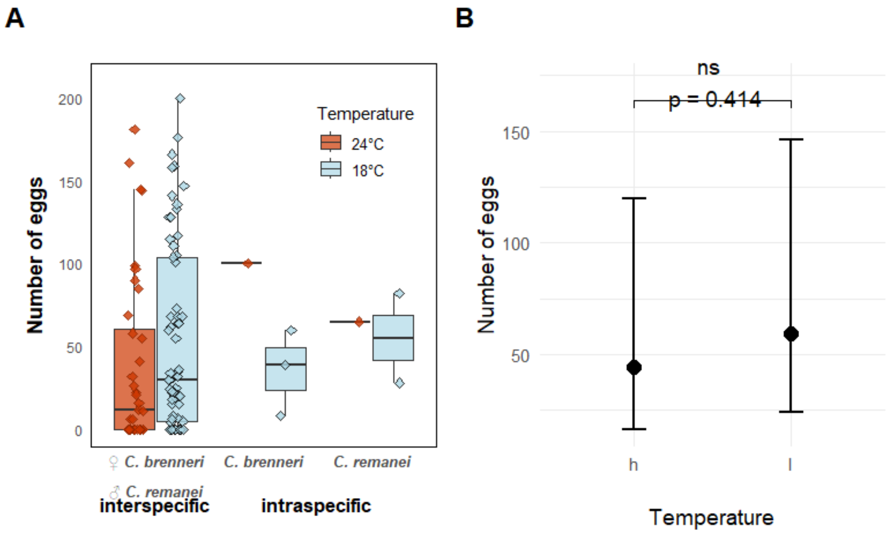

*Figure 1: A) Number of eggs layed at different temperatures B) Statistic model of effect of temperature on number of eggs*

## Offspring

Figure 2 shows the number of $F_1$ offspring produced in interspecific crosses between *C. brenneri* and *C. remanei* and intraspecific hybrids, comparing the effect of maternal species and temperature.

The interaction between temperature and cross type was not significant (p=0,94), so the final model included temperature and cross type as main effects. Temperature had a significant effect on total $F_1$ offspring number, LRT = 27.7, $p = 1.416 \times 10^{-7}$. The model-predicted mean number of offspring was higher at 18°C than at 24°C, with 12.38 offspring at 18°C compared with 7.01 offspring at 24°C. Pairwise comparison showed that offspring production at 18°C was 1.77 times higher than at 24°C, z = 5.03, p < 0.0001(Figure 3). Therefore, higher temperature significantly reduced total $F_1$ offspring production.

Intraspecific crosses with *C. remanei* mothers produced only a few observations at both high and low temperatures. Therefore, the sample size is too small to draw any reliable conclusions. These data could serve as a tentative control expectation for normal offspring production in interspecific crosses, but additional observations are needed before any statistical conclusions can be made.

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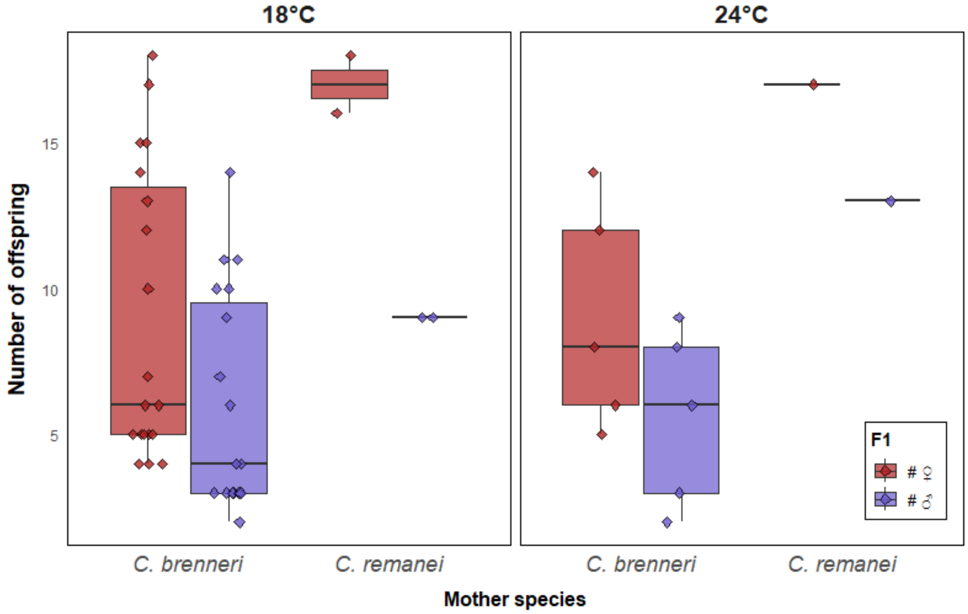

*Figure 2: Number of offspring at different temperatures.*

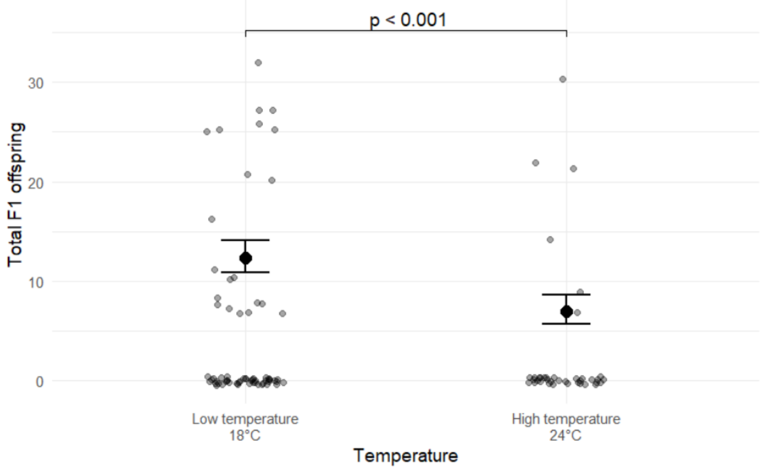

*Figure 3: Statistic model of effect of temperature on number of offspring.*

## Sex ratio

The predicted proportion of female offspring was similar at both temperatures with 0.602 at 24°C and 0.609 at 18°C (F = 0.137, p = 0.714). Cross classes were also analyzed as possible variable, the results showed no significant effect on sex ratio (F = 0.125, p = 0.724), with predicted female proportions of 0.599 in interspecific crosses and 0.617 in intraspecific crosses. Thus, neither temperature nor cross class significantly affected the proportion of female $F_1$ offspring.

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## Hybrid testing PCR

DNA extraction was performed several times on $F_1$ offspring of crosses that were considered successful, followed by gel electrophoresis. In some cases, two bands were observed, suggesting the offspring were of hybrid origin. However, the resolution and overall quality of the gels were poor, making the results difficult to interpret with confidence. Additional DNA extractions and gel electrophoresis runs were performed, but these did not produce clear results. In some cases, no DNA bands were detected, even in parental strains. This suggests that the problem may not have been limited to hybrid samples but could have been related to the DNA extraction protocol, PCR conditions, primer efficiency, or gel quality. The objective was to replicate the gel shown in Figure 4 (produced last year by a master student; Zouberou P., MSc thesis). This shows 2 clear bands in each $F_1$ hybrid.  Because this method was time-consuming and moved the focus away from the main objective of the experiment, PCR confirmation was eventually discontinued. Therefore, the molecular evidence for hybridization remained inconclusive.

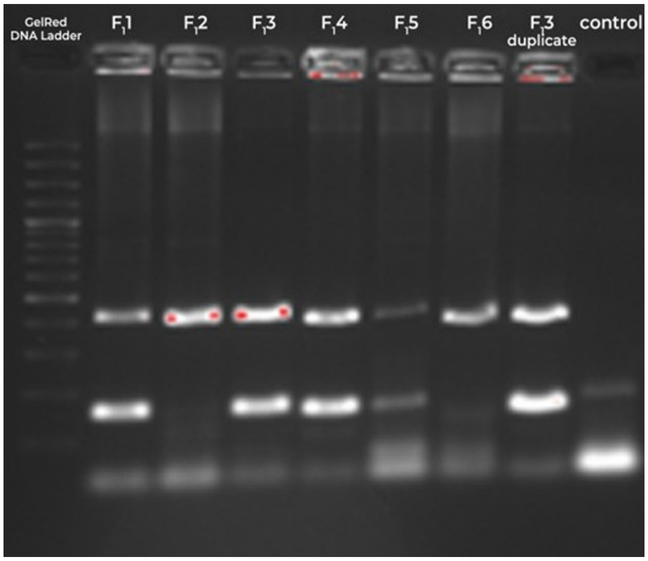

Figure 4: Agarose gel electrophoresis of the $F_1$ hybrids (Zouberou P., MSc thesis)

## Haldane’s rule

When a cross produced enough $F_1$ hybrids, 4 individuals were selected, usually 2 females and 2 males which were crossed with individuals of the opposite sex from each parental species. According to Haldane’s rule if one of the $F_1$ sexes was sterile it would be the males. Indeed, the $F_1$ hybrid males did not produce visible eggs (or offspring) when crossed with females of either parent. In contrast, $F_1$ hybrid females crossed with males from either parental species produced offspring. The number of eggs and offspring did not show any unusual outliers, and the individuals appeared to be in good condition. These observations suggest that female hybrids were fertile, whereas male hybrids were sterile. These crosses were performed 10 times however, the results were not quantified because this analysis was beyond the principal focus of the study. As a result, the sample size available for evaluating Haldane’s rule was too limited to generate a meaningful plot or support robust conclusions.

## Conclusion

This study investigated postzygotic reproductive isolation between *Caenorhabditis brenneri* and *Caenorhabditis remanei* at 18°C and 24°C. Interspecific crosses produced $F_1$ offspring, showing that reproductive isolation between these species is strong but not complete. Egg production was not significantly affected by

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temperature or cross type, suggesting that reproductive isolation does not mainly act at the level of egg laying. In contrast, $F_1$ offspring production was significantly lower at 24°C than at 18°C, indicating that higher temperature reduces offspring viability or developmental success. Temperature did not significantly affect the offspring sex ratio. Fertility tests showed that $F_1$ hybrid females remained fertile, whereas $F_1$ hybrid males appeared sterile, which is consistent with Haldane's rule. Overall, these results suggest that reproductive isolation between *C. brenneri* and *C. remanei* mainly involves postzygotic barriers affecting hybrid viability and male fertility, and that temperature can influence the strength of these barriers.

## Discussion

In contrast to a previous study (Sudhaus & Kiontke, 2007), it was possible to obtain offspring in interspecific crosses between *Caenorhabditis brenneri* and *Caenorhabditis remanei*. In addition, only $F_1$ hybrid males were sterile, a pattern consistent with Haldane's rule, which predicts that hybrid sterility or unviability should mainly affect the heterogametic sex.

Egg production was generally lower than expected across all crosses. Although *Caenorhabditis* females are often reported to produce large numbers of eggs or embryos under laboratory conditions. This suggests that reproductive output was reduced overall. Interspecific crosses tended to produce fewer eggs than intraspecific crosses, which is consistent with the expectation that crosses within the same species should be more reproductively successful than hybrid crosses. However, this difference was not statistically significant, meaning that the observed trend should be interpreted cautiously. The low sample size and the high number of failed crosses may have reduced the power of the analysis to detect differences between cross types.

A clear temperature effect was also observed. Crosses maintained at 24°C showed lower $F_1$ offspring production than those maintained at 18°C, suggesting that higher temperature reduced hybrid viability. This supports the hypothesis that heat stress can increase the expression of hybrid incompatibilities. The reduced mating success at higher temperatures may indicate that hybrid development is more sensitive to environmental stress than parental intraspecific development. Further data are needed to determine whether this effect is specific to hybrids or whether parental species are also affected by higher temperatures. Intraspecific crosses represent the expected reproductive output within the species. They are important to determining whether the reduction in $F_1$ offspring at 24°C is specific to hybrids or reflects a more general negative effect of temperature on reproduction and development.

The sex-ratio results did not follow the initial expectations. A stronger female-biased sex ratio was expected at higher temperature if male hybrids were more sensitive to heat stress. However, temperature did not significantly affect the proportion of female offspring. This result should be interpreted cautiously, as several crosses produced few or no offspring, which reduced the statistical power of the analysis. In addition, contamination on some plates may have made offspring counting and sex identification more difficult. Although adult males and females can usually be distinguished morphologically, contamination, crowding, young developmental stages, or poor visibility may have increased the risk of misclassification. The slower maturation of males was not studied or considered when calculating the sex ratio, which could explain why the observed ratio was not 0.5.

The observations related to Haldane's rule were important but should be considered preliminary. $F_1$ hybrid males crossed with females from either parental species did not produce visible eggs or offspring when crossed with females, while $F_1$ hybrid females remained fertile. This provides evidence consistent with hybrid male sterility. However, these crosses were mainly used as confirmation that the $F_1$ individuals were hybrids, rather than being analyzed as a separate dataset. In future experiments, hybrid fertility should be tested more systematically, with larger numbers of $F_1$ males and females, so that the validity of Haldane's rule can be tested statistically rather than only described qualitatively.

Molecular confirmation of hybrid identity using PCR was not successful. Although DNA extraction and gel electrophoresis were attempted several times, the results were unclear and could not reliably confirm the hybrid status of the $F_1$ offspring. In some cases, amplification failed even in the parental species, suggesting that the problem was not limited to the hybrid samples. Possible causes include poor DNA quality, unsuitable PCR conditions, old or damaged DNA polymerase, or problems with gel electrophoresis. Because troubleshooting PCR became too time-consuming and moved the project away from its initial objective, this approach was abandoned. As a result, hybrid identity was supported mainly by reproductive observations rather than molecular evidence.

Several limitations affected the quality of the dataset. First, sample sizes were inconsistent between temperatures and between intraspecific and interspecific crosses. The number of high-temperature crosses was lower because this treatment was added later, after the potential importance of temperature was identified from the literature. This imbalance makes comparisons between treatments less robust and may have influenced the statistical results. Future experiments should use a balanced design, with similar numbers of replicates for each temperature, cross type, and strain combination. The faster development at high temperatures was not considered when comparing with low temperature what makes it complicated.

Second, the dataset contained many zero values and failed crosses. Some failures may reflect true biological incompatibility between *C. brenneri* and *C. remanei*, but others were probably caused by technical difficulties. Worm picking was a major limitation, especially during the early stages of the experiment. Transferring one virgin female and several males required precision and care, as worms could easily be damaged, lost, or transferred with contamination. A large part of the early experimental period was therefore spent improving the picking technique before more consistent crossing success was obtained.

Overall, the results suggest that *C. brenneri* and *C. remanei* show strong but incomplete reproductive isolation. The production of $F_1$ offspring indicates that hybridization is possible, while reduced offspring production, temperature sensitivity, and apparent $F_1$ male sterility indicate the presence of postzygotic barriers. The data are consistent with Haldane's rule, but stronger evidence would require larger sample sizes, systematic fertility assays, and reliable molecular confirmation of hybrid identity. Future work should therefore focus on improving the experimental design, balancing the number of replicates across treatments, optimizing PCR or using alternative molecular markers, and treating hybrid fertility as a central part of the analysis rather than only as a confirmation step.

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Mata-Cabana, A., Romero-Expósito, F. J., Geibel, M., Piubeli, F. A., Merrow, M., &

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## Appendix 1: Tables with strain information

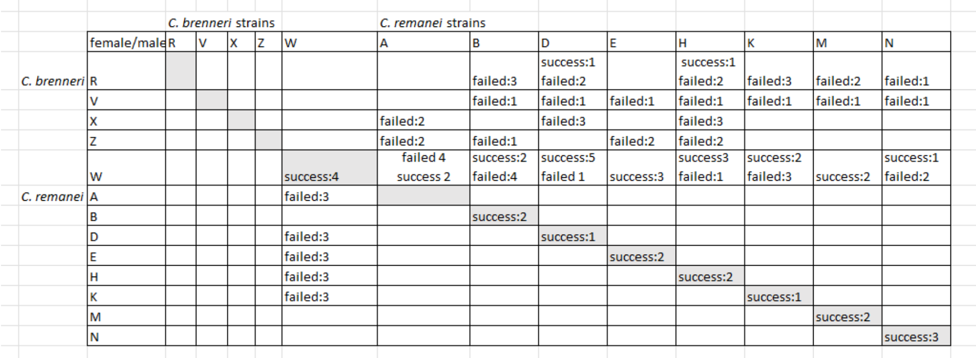
| Strain | Lab name | Locality description | Substrate |
|---|---|---|---|
| JU1084 | rC | Japan, Shizuoka p., Kakegawa | vegetal litter/mix |
| JU1086 | rB | Japan, Shizuoka p., Kakegawa | vegetal litter/mix |
| JU2179 | rD | Canada, Quebec, Montreal | rotting fruit (on ground) |
| JU2558 | rE | France, Grand Est, Illkirch | rotting fruit (on ground) |
| JU2744 | rH | Korea, Seoul | rotting fruit (on ground) |
| JU2794 | rI | Germany, Niedersachsen, Goettingen | rotting stem |
| JU2891 | rK | Turkey, Kilimli | rotting fruit (on ground) |
| JU3277 | rM | Czech Republic, Prague | rotting fruit (on ground) |
| TMR1001 | rA | Germany, Mittelsachsen, Praebschuetz | rotting fruit (on ground) |
| JU3419 | rN | Japan, Miyagi p., Akiu | vegetal litter/mix |

Table1: *C.remanei* strands used in Lab.

| Strain | Lab name | Locality description | Substrate |
|---|---|---|---|
| JU1323 | bP | India, Kerala, Poovar | rotting fruit (on ground) |
| JU1324 | bR | India, Kerala, Poovar | rotting fruit (on ground) |
| JU1397 | bU | Colombia, Medellin | rotting fruit (on ground) |
| JU1398 | bW | Colombia, Medellin | rotting fruit (on ground) |
| JU1815 | bX | French Guiana, Comte valley | rotting fruit (on ground) |
| JU1816 | bZ | French Guiana, Cacao | rotting fruit (on ground) |

Table2: *C.brenneri* used in lab

## Appendix 2: Tables all strains combinations and results



| | | *C. brenneri* strains | | | | | *C. remanei* strains | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | female/male | R | V | X | Z | W | A | B | D | E | H | K | M | N |
| *C. brenneri* | R | ▨ | | | | | | failed:3 | success:1<br>failed:2 | | success:1<br>failed:2 | failed:3 | failed:2 | failed:1 |
| | V | | ▨ | | | | | failed:1 | failed:1 | failed:1 | failed:1 | failed:1 | failed:1 | failed:1 |
| | X | | | ▨ | | | failed:2 | | failed:3 | | failed:3 | | | |
| | Z | | | | ▨ | | failed:2 | failed:1 | | failed:2 | failed:2 | | | |
| | W | | | | | success:4 | failed 4<br>success 2 | success:2<br>failed:4 | success:5<br>failed 1 | success:3 | success3<br>failed:1 | success:2<br>failed:3 | success:2 | success:1<br>failed:2 |
| *C. remanei* | A | | | | | failed:3 | ▨ | | | | | | | |
| | B | | | | | | | success:2 | | | | | | |
| | D | | | | | failed:3 | | | success:1 | | | | | |
| | E | | | | | failed:3 | | | | success:2 | | | | |
| | H | | | | | failed:3 | | | | | success:2 | | | |
| | K | | | | | failed:3 | | | | | | success:1 | | |
| | M | | | | | | | | | | | | success:2 | |
| | N | | | | | | | | | | | | | success:3 |

Table5: *Crosses summary.*

**Commented [PV10]:** Explain what success and fail means in terms of egg and F1 production

**Commented [PV10R2]:** Is this including Ezel's data? or just yours which are analysed in this thesis?

**Commented [SC10R3]:** I think ezel started it with me but we did stop early do focus on Bw and I dont know if she added more

This table presents the first step of the study, in which all possible strain combinations were tested. A cross was considered successful when clear egg production and viable offspring were observed. Failed crosses were defined as crosses that showed no egg production or offspring production after three days. If a cross failed due to other reason like female or male dying, contamination, bad picking or worms escaping was retried, especially in strains still showing a success chance. Not all strain combinations were tested equally, because the probability of success using female *C. brenneri* with the remaining *C. remanei* strains was considered low if previous combinations had already failed. Therefore, a decision was quickly made to focus on bW females for hybrid crosses in the subsequent part of this thesis.

## Appendix 3: Code availability

The script used for data processing, statistical analysis, and figure generation is available in the following GitHub repository:

**Commented [PV11]:** Nice to use github, also add the data so people can use the script!

SebastianCapelle/R-script-Bachelor-thesis: Code used for data processing and analysis in my bachelor thesis.

*The repository contains the complete analytical script and data used in this thesis.*

## Use of GenAI

| **Applications** | **Tool** | **What did you do and where in the document.** |
|---|---|---|
| X Language support | Chatgpt | To verify English spelling and grammar in each text. |
| O Search engine | | |
| X Literature research | Claude | To confirm there was no *C.brenneri* temperature effect article.Used for in the introduction. |
| O Help with short input (e.g. completing sentences, text assistance) | | |
| X Generating programming code | Claude | To improve graphs and scripts. It helped me to solve errors or find the wanted script code. Was used in results section. |
| O Generating new research ideas | | |
| O Generating text blocks | | |
| O Other applications Explain: | | |