Effects of the gene *transformer* on sex determination, development and behavior in *Leptopilina clavipes*

Name: Charel Jager

S-number: S3499340

Date: 8-4-2022

Study: BSc Biology, ecology & evolution

Partner: Rosan Favot (MSc)

Supervisor: dr. E. Geuverink

Abstract

Many of the *L. clavipes* parasitic wasps are infected by the *Wolbachia* bacteria, changing the reproductive strategy from arrhenotoky to thelytoky. Yet how this occurs in this species is yet unknown. By identifying the influence of *Wolbachia* on this system, first it needs to be studied how the sex determination occurs in the arrhenotoky strategy. By knocking-down one of the genes involved in the sex determination, the *transformer* gene, it is expected that a change will occur in the offspring. In the study the knock-down of the asexual line (LS1/LS3) was proven, but the results in the offspring were not as expected. While in the sexual line (CA1) the knock-down was not proven. The *Wolbachia* bacteria has a large effect on the reproductive strategy, but it appears it might have an effect on the sex determination of the offspring as well.

Introduction

The amount of insect species is enormous and it is a highly successful group, with species in both terrestrial and water habitats. This specialist group has a wide array of sex determining systems (Blackmon et al., 2017). In insects the haplodiploid system is not uncommon, it is largely present in the Hymenoptera, Phthiraptera and Thysanoptera. But even within the haplodiploid system there is a large amount of variability, for instance the origin of the sexes.

Insects are often sexually reproductive organisms. Commonly the reproduction mode of haplodiploids is arrhenotoky, where the female can produce unfertilized eggs which develop into haploid males or fertilized eggs which produce diploid females. Nevertheless, haplodiploids can use as reproductive strategy thelytoky. Which is an asexual way of reproducing, where unfertilized eggs to develop as complete females (Pannebakker et al., 2004).

Approximately 20% of all insect species is infected by the *Wolbachia* endosymbiont (Werren et al., 1995). The *Wolbachia* bacteria has many influences on its host, one of them is the reproductive manipulations for instance creating haplodiploids whom use thelytoky as reproductive strategy (Hoffmann, 2020). The *Wolbachia* has been present for thousands of years and is both vertically and horizontally transferred and because of that might be selected to increase host survival and reproduction (Bordenstein & Werren, n.d.).

The parasitic wasp *Leptopilina clavipes* is a Hymenoptera species which can contain the *Wolbachia* bacteria. When the *L. clavipes* contains *Wolbachia*, the reproductive strategy changes from arrhenotoky to thelytoky. In the arrhenotoky reproductive strategy of the *L. clavipes* there is a lot unknown how the gender differentiation occurs. It has been shown that, similar to *Nasonia vitripennis*, in the sex determination the *transformer* (*tra*) gene has an important influence (Beukeboom & van de Zande, n.d.). While in *N. vitripennis* it is known that the instructor gene *wasp overruler of masculinization* (*wom*) gene starts the cascade of the sex determination, with as most important genes the *tra* and *tra2* genes (Zou et al., 2020). In the *L. clavipes* the identity or identities of the instructor gene(s) have not been described yet.

In the *L. clavipes* it has been studied that one of the genes involved in the sex determinations is the *tra* gene. It appears that the *tra* gene has multiple splice forms (figure 1) which can be attributed to the different sexes, therefore they are labeled tra^{F} (female) and tra^{M} (male). The tra^{F} splice variant has less exons than tra^{M}, nevertheless the tra^{M} gene codes for an early STOP-codons, resulting in a shorter TRA-M protein compared to the TRA-F protein (Geuverink et al., 2018). These *tra* genes, will influence the next step in the cascade. Presumably the *tra* genes will cause a splicing difference in the *double sex* (*dsx*) gene, causing a splicing towards either the female or the male form.

The *tra* gene expression appears to be upregulated in the young stages of the individuals in the presence of *Wolbachia* (Geuverink *et al*, in prep). Therefore, to identify the expression of *tra* can be best measured in embryos or ovaries with maternally provided mRNAs.

In order to identify how *Wolbachia* can influence the reproductive strategy of *L. clavipes*, it is important to study how the sex determination occurs in the sexual reproduction (arrhenotoky) of the *L. clavipes*. By knocking down the *transformer* gene a change is expected to be found in the offspring of the knocked-down female wasp. By knocking-down the *tra*-gene, it is expected that the *dsx* splicing is switched towards the male form. Which would be visualized in the forming of (more) males, of most likely the diploid form.

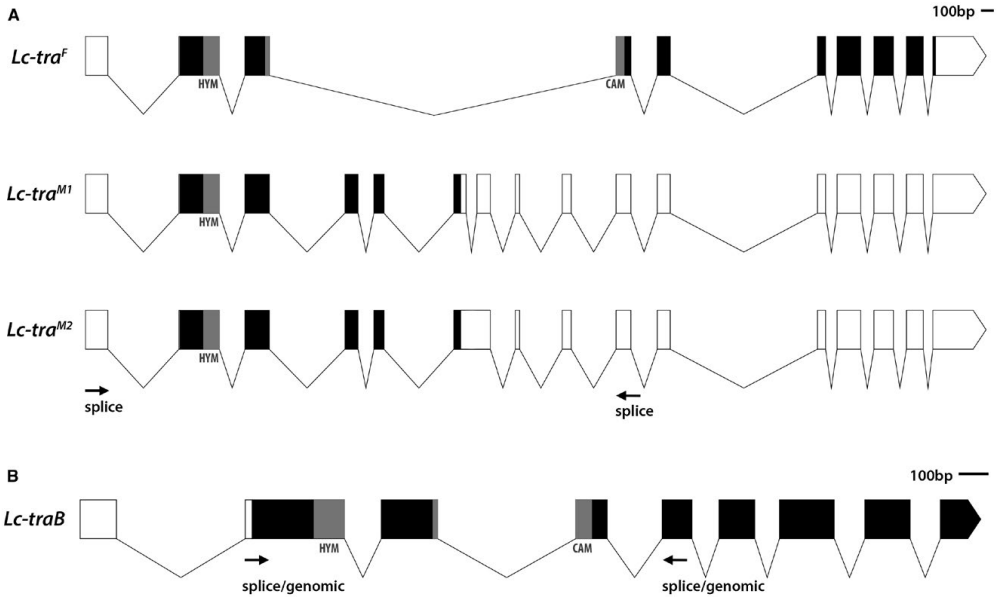


Figure 1: Splice variants of the *transformer* gene (Geuverink et al., 2018)

Materials and methods

Insect culturing

Sexual strains *Leptopilina clavipes*, CA1, which parasitize on *Drosophila melanogaster* and the asexual strains *Leptopilina clavipes* LS1 and LS3, which parasitize on *Drosophila virulens*, LS1 and LS3 are considered the same strain. The sexual females are allowed to mate with males from the same lines and all females will parasitize young *Drosophila* larvae in 25^{o}C on agar soil with diluted yeast.

The *L. clavipes* pupae were removed while in the red-eyed stage, from the *Drosophila* pupae host and glued on their back to an object glass. Of CA1 strain 181 individuals were injected and of the LS1/LS3 strain a total of 231 individual females were injected. Afterwards placed in 25^{o}C. When the wasps turn completely black and start moving, they are counted and placed with honey water in 12^{o}C.

RNAi injection

The dsRNA sequence provided kindly by Rosan Favot (MSc biology) and dr. E. Geuverink. 6µg/µL dsRNAi diluted to 4,8µg/µL with red food dye into abdomen of the *L. clavipes*. Injections performed with Green fluorescent protein (GFP) RNAi, MQ and *transformer* (*tra*) RNAi. GFP injections in CA1, n=19 and in LS1/LS3 n=48, MQ injections in CA1 n=71 and LS1/LS3 n=45 and *tra* injections in CA1 n= 91 and LS1/LS3 n=93. The individuals injected are G0

RNA extraction and cDNA synthesis

RNA extracted with TriZol (Invitrogen, Carlsbad, CA, USA), according to manufacturer's protocol from ovaries of adult females (G0) after parasitizing (24h) an overload of larvae (F1). Similarly, RNA was extracted from all available whole larvae (n=10) after being parasitized by *L. clavipes* for 18h, of both the sexual and asexual lines. The ovaries were scored upon sight in categorical values from 1-4. RNA was reversed transcribed to cDNA with Thermoscientific RevertAid H Minus First Strand cDNA synthesis Kit (Thermofisher Scientific; REF: K1632, Lot: 00823963) according to manufacturer's protocol.

Identification of *tra* gene

With PCR the *tra* gene was located, identified and compared to the reference genes RPL13 Forward 3'-AGAAACAAATCCGTCGAATCTC-7' Reversed 3'-CCCTTCTTCGGCTTCTTCTC-7' and ADEK Forward 3'-ATGAAAGACGACGTGACAGG-7' Reversed 3'-TCGACTAGAGGTTGTGTTTGTG-7'. The reaction was performed at 95^{o}C for 3min, 45cycles of 95^{o}C for 30s, 57^{o}C of 30s, 72^{o}C for 30s and a final extension of 72^{o}C for 7min. And the transformer splicing variances tested with traA_spliceF1 Forward 3'-CATCCAGAGACAGACGATCC-7' traA_splice_R1 Reversed 3'-TACTTCTCGATGTTCACCTTCC-7'. The reaction was performed at 95^{o}C for 3min, 45cycles of 95^{o}C for 30s, 57^{o}C of 30s, 72^{o}C for 120s and a final extension of 72^{o}C for 7min. The amplifications were visualized on a 1.5% agarose gel of 1x TAE with SERVA DNA Stain G (SERVA serving scientists; cat 39803) with GeneRuler 100bp (ThermoFisher Scientific; cat: SM0241) and TriTrack DNA Loading dye (Thermofisher Scientific; cat: R1161).

With RT-qPCR the quantity of the *tra* gene was studied, by using the same reference genes RPL13 and ADEK and the *tra* gene with tra_embryo_qPCR_f4 Forward 3'-GAGTACATCATCGAGACCAACAG-7' and tra_embryo_qPCR_r4 Reversed 3'-GACTGAAACGAGGATTAGGAGGA-7'. Samples created with PerfeCTa SYBR Green FastMix ROX (QuantaBio; cat 95073, Beverly, MA) and the reaction was performed at 95^{o}C for 3min, 45cycles of 95^{o}C for 15s, 56^{o}C of 30s, 72^{o}C for 30s and a dissociation curve of at 95^{o}C for 15sec, at 60^{o}C for 1min, at 95^{o}C for 15s and at 60^{o}C for 15s.

Scoring ovaries

Randomly 10% of each group were chosen after 24h parasitizing, of which the ovaries were removed and scored on appearance. Whether there were 1 or 2 ovaries present, where they covered in a white matter. If the ovaries were both present without abnormal view, they were scored a 4. If there was only 1 ovary present with abnormal view, they were scored a 1. The ratio of this was created to a percentage.

Data-analyses

Statistical analysis performed using the program R. The data, when normally distributed, was tested with a 1-way ANOVA test. When data was not normally distributed a Kruskal-Wallis test was performed. Statistics which significant differences were placed by the data, P-values < 0,05 were considered significant. Graphs were formed with the software PRISM.

Results

Mortality due to injection

To create control groups and treatment groups, female wasps in red eyed stage were injected with either MQ, GFP RNAi or *tra* RNAi. The groups sizes differed due to limiting factors such as high mortality rates. In table 1 is the total amount of injected individuals (No), the number of deceased individuals (Deceased) and the survival percentage per group. The ovaries were scored on appears in a small sample size. There is a trend visible in the asexual line, where the injections with GFP caused a death rate of almost 92%. Besides this, there is no significant changes visible between the mortality of injections with MQ and TRA.

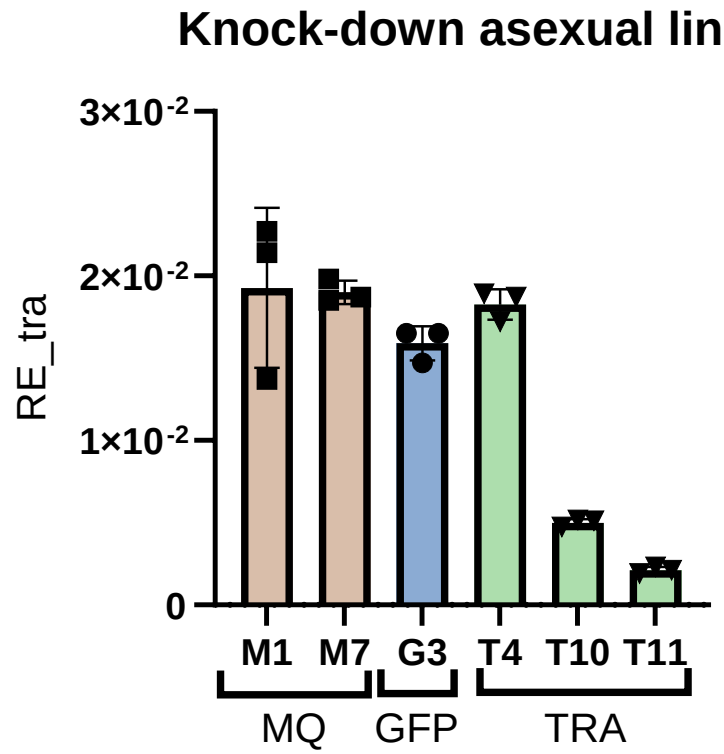
Table 1: Total amount of injected individuals and their survival percentage after injection.
The ovaries were scored based on appearance and the deceased were counted approximately 2 weeks after the last individuals were marked alive. The table shows a large decrease in survival percentage in asexual line of the GFP injected. In the other groups no differences were found.

sexual/asexual	Injection_with	No	deceased	Survival (%)	Ovaries (%)
sex	GFP	19	7	63.15789474	85.71
sex	MQ	71	38	46.47887324	77.77
sex	TRA	91	39	57.14285714	89.47
asex	GFP	48	44	8.333333333	66.66
asex	MQ	45	32	28.88888889	93.75
asex	TRA	91	51	43.95604396	87.5

Knocking-down the *transformer* gene in adult female

In order to identify whether a knockdown of the *tra* gene was induced, a RT-qPCR was performed on a limited amount of the injected samples. Of the survived individuals after injections approximately 10% were used for the RT-qPCR study, all sexual individuals had the time to mate and all sexual and asexual individuals had 24h time to parasitize. In figure 2 the different numbers within each group are the biological replicates (grouped by color) and the different points within each sample are the technical replicates, all results are standardized upon the RPL-13 gene. In figure 2A the results of the asexual line are visible. As shown in the control groups (MQ and GFP) the average amount of gen-expression is $2x10^{-2}$. A similar gene-expression can be found in sample T4. In sample T10 the gene-expression has been lowered to $5x10^{-3}$ and in T11 $2x10^{-3}$. In figure 2B the results of the sexual line are visible. The MQ control group was removed due to technical difficulties. The gene-expression of the GFP control group and the TRA group were in the same range around $4{,}5x10^{-5}$. The results showed a positive signal for the knock-down for *tra* in some samples.

A



B

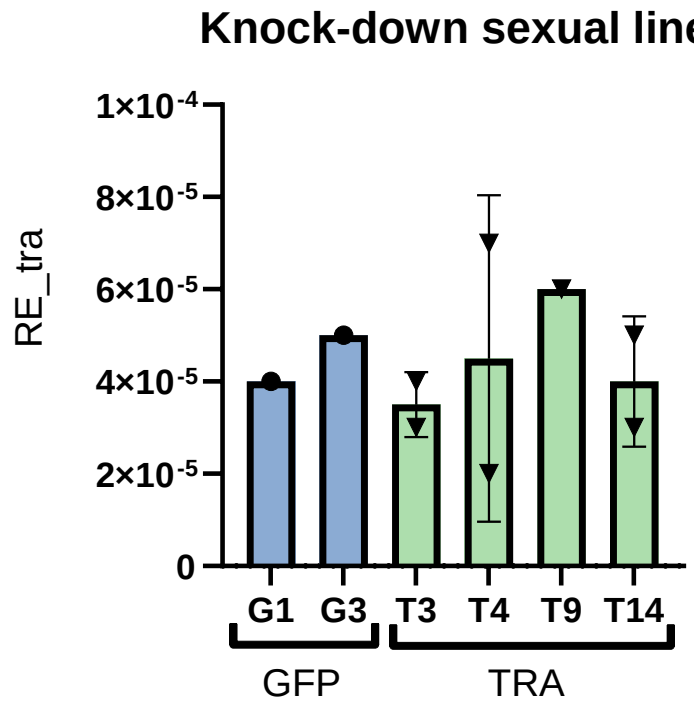


Figure 2: Testing the gene-expression with RT-qPCR in the *tra* knock-down groups compared to the control groups MQ and GFP standardized against the gene RPL13. Approximately 10% of the survivors of the injection were used for the RT-qPCR, all sexual individuals had mated and all sexual and asexual individuals had 24h time to parasitize. This shows a decrease in the gene-expression the asexual line in 2 of the 3 *tra* knock-down compared to MQ and GFP. And no decrease in the sexual line.

Offspring F1 survival

To identify the offspring sexes formed and to see the behavior changes after injections, the offspring were counted as well as the sex ratio. This numbers show all the individuals whom survived injections, including the samples used for the RT-qPCR. After the G0 had parasitized an overload of larvae, the number of offspring per individual female per group was counted. Therefore, all the datapoints are the number of individuals per individual wasp. The parasitizing rate was never 100%, therefore *drosophila* flies were found in every sample. Therefore, the number of hosts was not a limiting factor. In figure 3.A the amount of asexual adult offspring is shown, there are no offspring found in the GFP group and only 2 MQ injected females produced offspring and only 5 *tra* injected females produced offspring. In figure 3.B the amount of sexual adult offspring is shown, there are offspring found in each group. Only a small number of injected females did not produce any offspring.

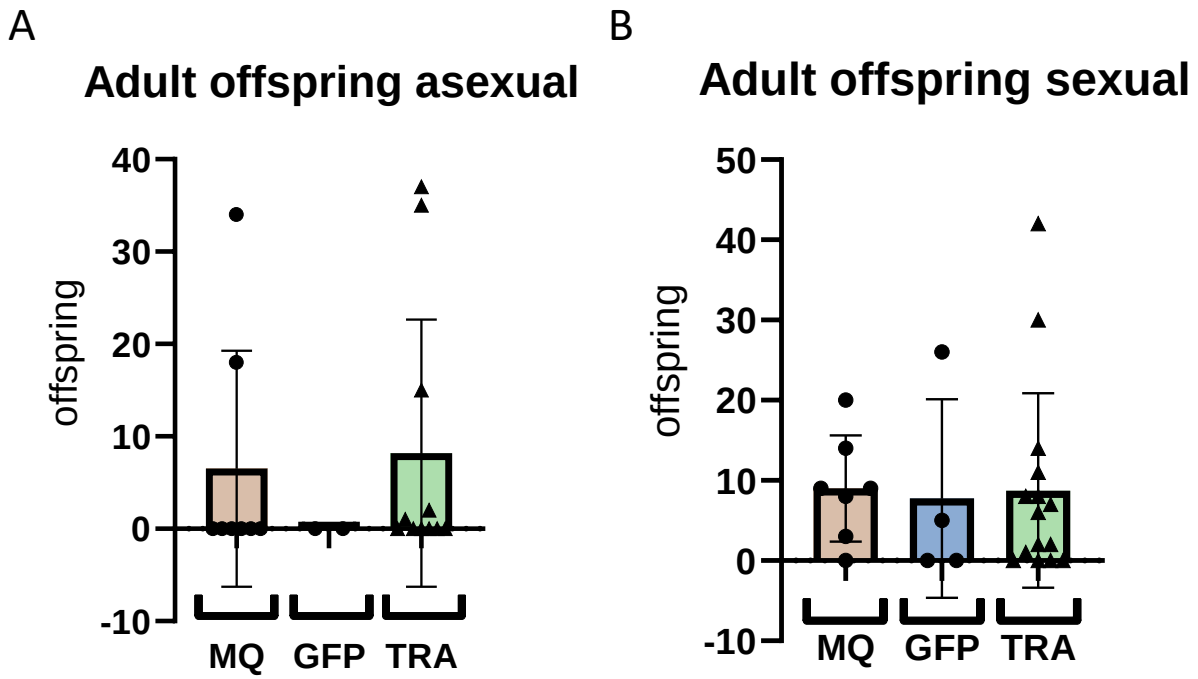


Figure 3: Measuring the number of offspring produced by the individual wasps, whom were injected with treatments, compared within the group a trend is visible. In the 2.A the asexual line shows that in most cases no offspring were formed, specifically the GFP. In the sexual line 2.B the number of offspring produced is about the same between each group. In this graph all individuals whom survived the injections were given the opportunity to mate (sexual) and parasitize, including the samples used for RT-qPCR.

Sexes production

With the knock-down of the *transformer* gene, it is expected that a change in sex ratio will be found. With the asexual strain normally only producing females, it is expected that they will produce males instead. While in the sexual strain the mated female should produce diploid females, it is expected should now produce diploid males. In the asexual line, all offspring found were identified as female due to short antennas. In table 2, the offspring of the sexual line is visible. In the G0 group the amount of injected fertilized females per group is visible. The F1_male/G0 shows how many males are produced per G0 female, and the F1_male/F1_total shows the percentage of the offspring are males. The table shows that overall, the MQ injected fertilized females have the highest production

of males and the GFP and *tra* injected fertilized females have similar number of male offspring. In figure 4, the injected fertilized sexual females whom had not reproduced are left out. Comparing the offspring of the *tra* injected females to the control injected females. There is no trend nor significant difference in the number of male offspring produced.

group	G0_individual	F1_male	F1_male/G0	F1_male/F1_total
GFP	4	14	3.5	0.451612903
MQ	7	58	8.285714286	0.920634921
TRA	15	78	5.2	0.595419847

Table 2: The difference in production of male offspring in fertilized injected females between groups. The G0 females all had time to mate and to parasitize an overload of hosts. The table shows no difference between the production of male offspring between GFP and *tra*. The MQ injected females have a higher number of male offspring.

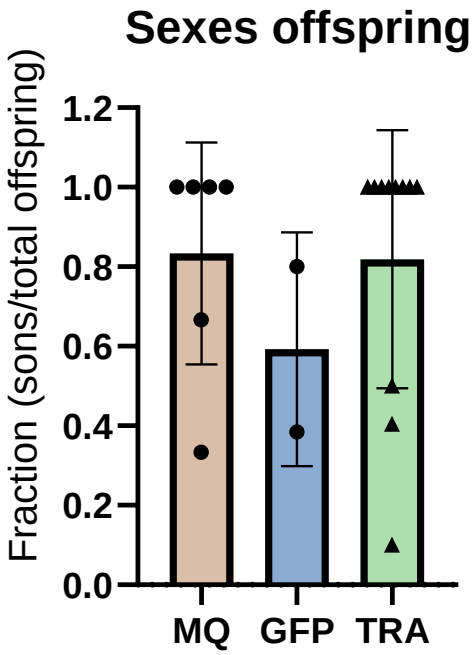


Figure 4: Ratio of sons (F1) born out of injected fertilized females in the sexual line. In the graph the females whom did not produce any offspring are left out.

Behavioral changes between groups

When knocking-down the *transformer* gene in developing females, differences in behavior might occur. Therefore, the mating behavior of the sexual line was observed and the parasitizing behavior of both lines.

In the sexual lines, 6 G0 females whom mated, did not produce female offspring. Overall, it was scored that 13 females mated and 19 produced offspring of which 7 produced females. Not all females whom mated did produce offspring and not all whom mated did produce female offspring.

In the asexual line, 7 wasps produced offspring of only 1 was scored as parasitizing behavior.

Measuring F2 offspring

In order to identify the parasitizing behavior of F1 offspring of G0 with *tra* injected females in the asexual lines, the *L. clavipes* eggs inside the *Drosophila* larvae were identified. The F1 females had 4h to parasitize the larvae and the eggs had 18h to develop inside the host. By performing a control PCR with the RPL-13 gene on whole larvae, it was tested if the F1 females had parasitized the larvae and therefore were females. In figure 5 the results of the PCR are visible, with a 100bp ladder and in line 1 and 2 are the control samples whom are not injected nor offspring of injected G0. Line 3, 4 and 5 contain biological replicas of F2 offspring of *tra* injected G0.

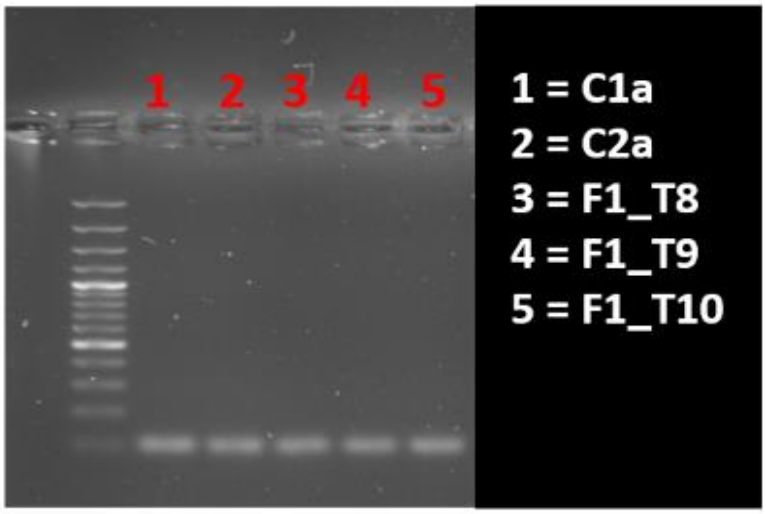


Figure 5: The F1 females had 4h to parasitize the larvae and the eggs had 18h to develop inside the host. The larvae showed clear prove of eggs laid by the F1 asexual females. The RPL-13 should only have been visible if the *L. clavipes* had parasitized the larvae.

Discussion

Overall, the study would be improved with a larger sample size and more timepoints to test upon. More ideal would be to also be able to knock down different genes in the sex determination cascade. In *N. vitripennis* there is the possibility of changing eye color depended on the chromosome amount, so for instance haploid males could have grey eyes while diploid males have red eyes. Nevertheless, to create these models, first it has to be tested whether diploidy is a possibility in the *L. clavipes*. Up to this point there is no prove for viable diploid males in the *L. clavipes* or haploid females.

In creating the knock-down, it was shown that the knock-down was not succeeded in the sexual line. Nevertheless, in the sex ratio test there were samples whom had more female offspring than male offspring. It should be tested upon more to find the reason why the knock-down did not succeed here. In the study of (Geuverink et al., 2018) differences between arrhenotoky and thelytoky where shown, one of the results showed a difference in splicing variants at different time points. Perhaps the ds RNAi of the *transformer* gene could knock-down the effects of the gene in the asexual (thelytoky) but not in the sexual (arrhenotoky). In this case the RNAi should be created more specifically on the sexual strain.

With in the F1 no offspring being formed in a large group, the question arises whether the knocked down offspring did have a chance to develop to adult wasps. In the offspring count it was found that in some pupae full grown wasps were found, but they did not emerge. Nevertheless, they had all matured to that stage, younger less matured wasps were hardly found. Therefore, it is assumed that the wasps could not emerge out of the pupae due to differences in humidity.

In the asexual line the GFP injected females did not thrive at a similar level as the MQ control or the *tra* treatment. It appears that most females injected with GFP died at a young stage or did not reproduce at all. GFP might therefore not be the best control group to identify the response of the immune system on RNAi. It appears as if the interference RNA has found a different gene to interfere with, which might have an effect on the lethality of the *L. clavipes*. In the asexual line a clear knock-down was achieved, nevertheless this knock-down was not passed along to next generations. The F1 created out of G0 females injected with *tra* RNAi, still appeared as females with shorter antennas and could also still parasitize hosts. There are cases known where gynandromorphs are formed, for instance in the *N. vitripennis*. Yet these gynandromorphs are sterile and can't reproduce. With the F1 group being able to parasitize hosts, there is no chance that here gynandromorphs were formed.

The reason for why this knock-down is not passed along is still unknown. But multiple hypothesis could explain this effect. Firs of all the presence of *Wolbachia* is the most reasonable explanation. With *Wolbachia* having the ability to interfere in the cell division during mitosis it is possible that not only does it interfere it might bring important genes with it to the offspring to ensure the production of females. Secondly perhaps the *transformer* gene is not passed along from mother to offspring, but the instructor gene is causing the offspring to create its own *transformer* gene. Lastly the influence of time is not taken into account. In recent studies (Geuverink *et al*, in prep) it has shown that the *transformer* gene is abundantly present in the ovaries, but decreases rapidly in the rest of the wasp body.

Conclusion

In this study it was attempted by knocking-down the *tra* gene in both a sexual line (arrhenotoky) and an asexual line (thelytoky) to gain knowledge about the sex determination in the *L. clavipes*. It was expected that in the sexual line, more males would be found. Due to the fact that the unfertilized offspring would continue being haploid males but the fertilized offspring would become diploid males. In the asexual line, there is expected that only diploid males would be formed. In the sexual line, no knock-down of the *tra* gene was achieved. In the offspring formed no evidence was found of the *tra* knock-down. In the asexual line a clear knock-down was achieved. By following an individual with a knock-down of the *tra* gene, it was shown that the offspring were still all female. These F1 females also had the ability to reproduce and parasitize hosts. Because the sexual line did not show a knock-down of the *tra* gene at all, the effect of the *transformer* gene in the sex determination can not be anticipated. When the knock-down is present in the parent (G0) it does not appear to be passed to the offspring. This could display the influence of *Wolbachia*, perhaps *Wolbachia* can bring the *transformer* gene along the offspring.

References

Beukeboom, L. W., & van de Zande, L. (n.d.). *Genetics of sex determination in the haplodiploid wasp Nasonia vitripennis (Hymenoptera: Chalcidoidea)*.

Blackmon, H., Ross, L., & Bachtrog, D. (2017). Sex Determination, Sex Chromosomes, and Karyotype Evolution in Insects. *Journal of Heredity*, *108*(1), 78–93. https://doi.org/10.1093/JHERED/ESW047

Bordenstein, S. R., & Werren, J. H. (n.d.). *Do Wolbachia in¯uence fecundity in Nasonia vitripennis?*

Geuverink, E., Kraaijeveld, K., van Leussen, M., Chen, F., Pijpe, J., Linskens, M. H. K., Beukeboom, L. W., & van de Zande, L. (2018). Evidence for involvement of a transformer paralogue in sex determination of the wasp Leptopilina clavipes. *Insect Molecular Biology*, *27*(6), 780–795. https://doi.org/10.1111/IMB.12522

(Geuverink *et al*, in prep).

Hoffmann, A. (2020). *Wolbachia*. https://doi.org/10.1016/j.cub.2020.08.039

Pannebakker, B. A., Pijacker, L. P., Zwaan, B. J., & Beukeboom, L. W. (2004). Cytology of Wolbachia-induced parthenogenesis in Leptopilina clavipes (Hymenoptera: Figitidae). *Genome*, *47*(2), 299–303.

Werren, J. H., Zhang, W., & Guo, L. R. (1995). Evolution and phylogeny of Wolbachia: reproductive parasites of arthropods. *Proceedings. Biological Sciences*, *261*(1360), 55–63. https://doi.org/10.1098/RSPB.1995.0117

Zou, Y., Geuverink, E., Beukeboom, L. W., Verhulst, E. C., & van de Zande, L. (2020). A chimeric gene paternally instructs female sex determination in the haplodiploid wasp Nasonia. *Science*, *370*(6520), 1115–1118. https://doi.org/10.1126/SCIENCE.ABB8949/SUPPL_FILE/ABB8949_ZOU_SM.PDF