How I Met Your Mother: interspecific parasitoid male host specificity

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Abstract:

Nasonia is a genus of parasitic wasps, consisting of 4 species. *N.vitripennis, N.giraulti, N. longicornis and N. Oneida.* Previous research shows that not only females but also males display host specifity. This research controls for and builds further on this research and is divided into three different topics. These topics are related to male host specifity in this genus of wasp. The four topics are: Host specificity on same species, Host specificity between species and the Genetic region of host specificity in males. The setup used in the experiments is an arena setup, where the virgin males got to choose between 2 or more choices. The arena was divided into 8 or 6 parts with alternating options. The results showed consistent and inconsistent results with research previously done by Prazapati et al.. It was found that male *N.vitripennis* showed a significant preference for the parasitized pupae, but contrary to their work in this research it was found that males could not distinguish between mixed and all male brood. Furthermore it showed that when the male "chooses" a mixed brood pupae it does it at random rather than maximizing the proportion of females inside. For the interspecies host specifity it did not show any significant results for its own or other species. For the BKBWG strain the results were all insignificant except for the white eyed males having a significant preference for parasitized pupae.

Introduction

Nasonia is a genus of parasitic wasps, consisting of 4 species. *N.vitripennis, N.giraulti, N. longicornis and N. oneida* (Michael et al, 2010) . A parasite is: "an organism that lives on or in a host organism and gets its food from or at the expense of its host" (*CDC - Parasites - About Parasites*, n.d.). In the case of *Nasonia* these hosts are many of the blowfly species. *N.vitripennis* is a generalist species, meaning that they can parasitize multiple species of host. The other three *Nasiona* species are specialists that have a strong innate preference for one host genus. The *Nasonia* species females use their host to lay eggs A hole is drilled in the puparium (the outside shell of the host) and the eggs are deposited via the ovipositor of the female in the space between the puparium and the host pupa. Accompanying the eggs is a venom that kills the host pupa. The venom creates an environment in which the *Nasonia* larvae can feed on the host and grow (Michael et al, 2010).

Nasonia females only mate once. Therefore there is strong sexual competition between males to be the first to mate with the female. This competition gives rise to different sexual strategies across different stages of life. The sexual life cycle of *Nasonia* is as follows:

1. The fertilized eggs are in the host pupa on top of the host larvae;
2. The larvae of *Nasonia* grow while feeding on the host's body;
3. Males mature earlier than females and emerge through the emergence hole
4. Males travel to others hosts to look for possible female mates;
5. Females mature and appear out of the emergence hole to find a suitable male. Most females of *Nasonia* mate on or within their natal host patch, or don not mate;
6. When the male is liked by the female it chooses to mate with him and her eggs are fertilized. After this first copulation, if the male performs post-copulatory behaviors, she will refuse to mate with any other male **,**
7. Female looks for a suitable host to place her eggs in via her ovipositor (Prazapati et al, 2022).

The genus *Nasonia* falls under the insect order *Hymenoptera* and thus the sex of the individual is determined by haplodiploidy. This means that females have a double set of chromosomes and a male has a single set of chromosomes. Virgin females produce all male broods and mated females produce a mix of males and females, with the female ratio often being higher (Michael et al., 2010).

The sexual reproductive success of a *Nasonia* male is limited by the amount of fertile females it can mate with. As female mating is often restricted to their natal host patch, males look for hosts in their vicinity and defend it until the female emerges through the emergence hole. Defending the hole increases their chance of being the first to mate with the newly emerged females. But other hosts in the natal patch may be parasitized, and so also have potential mates. Additionally, with haplodiploidy being their sex determination mechanism, it is possible that a host is filled with only male offspring from a virgin mother. Hosts may also have a different species of *Nasonia*, as several species may use a single natal patch (bird nest). An interspecies interaction would not result in a viable lineage. It would thus not be beneficial for the male to defend a host filled with offspring it can not mate with, as this is a waste of energy.

It has been known that females of the *Nasonia* genus have host specificity (Prazapati et al, 2022). But a research done by Prazapati et al. shows that not only females but also males exhibit host specificity. The research showed that all of the species of nasonia can distinguish parasitized pupae from unparasitized pupae. But out of all 4 species only the males of *N. vitripennis* can distinguish between all male brood and a mixed male/female broods. Uncovering a previously unknown reproductive strategy in males of one of the most studied parasitic wasps.

This study will focus on the same topic of male host specificity. Four different things are going to be discussed;

- Host specificity on same species
    - Parasitised versus unparasitised
    - Female brood versus all male brood
    - Proportion of females/amount of females within the host
- Host specificity between species
- Genetic region of host specificity in males

The first and second topic is a control for previous research. Done by Prazapati et al. the corresponding question that needs to be asked is:

Do male N. vitripennis of the strain ASYMCX show a significant preference for parasitized fly pupa containing females.

The third topic is about the proportion of females in the brood. Because competition between males is so strong and male sexual success is measured by the amount of females it mates with. It could be an additional sexual strategy for the males to optimize their chances and choose a parasitized host pupa containing a high proportion of females. And put more effort into defending it. This yields the second question asked in the study.

Do male N. vitripennis show a significant preference for pupae containing a higher proportion of females.

It is important for the species of *Nasonia* to mate with their conspecific, as creating hybrids can be detrimental for the fitness of their offspring. The offspring created could be non viable, sterile or be less fit than other interspecies mated individuals (Cohen et al, 2021). *N. Vitripennis* is a generalist species meaning that they can lay their eggs in multiple host species, the other three Nasonia species are specialist, meaning that they have a smaller niche amount of hosts where they lay their eggs in. Because *N. vitripennis* could also lay eggs in the same host as the other three, and because it is not possible to see from the outside which species is inside, the question asked in this paper is:

Can male N. Vitripennis have a significant preference for pupa containing conspecific larvae or the larvae of other species.

The fourth topic is about the location of the host specificity gene. Research done by Desjardins et al. showed that the locus of host specificity in females lies very close to the locus of eye color. In the *N.vitripennis* BKBWG strain there are two sorts of eye colours, white and peach. The white eyed individuals have the specialist genes (*giraulti*) and the peach eyed individuals have the generalist genes (*vitripennis*). The bkbwg region controls host specificity in females. The peach, small wing phenotype is from the generalist *N. vitripennis*, and the white eye, small wing phenotype is from the specialist *N. giraulti*. The question that needs to be answered is whether this region also controls the female detection phenotype in males. If it does, then the peach eye males should be able to, like the male *N. vitripennis*, and the white eyes should not, *like N. giraulti*. This reared the last research question:

Is the genetic location for parasitation detection on the same locus as peach/white eyes?

Methodology:

During this study *N.vitripennis* of the strain ASYMCX and BKBWG were used. *N.oneida N.longicornis: N.giraulti*. As a host the pupa of the species *Calliphora* were used.

To obtain all male broods, a virgin female was placed into a testing tube with three *Calliphora* pupae (Host). These test tubes were subsequently placed into a 25 degrees celsius incubator for one day and then moved to the 30 degrees celsius incubator. Every day progress was tracked until the *Nasonia* pupae reached the "black stage". After this stage was reached the test tubes were placed into a 4 degree celsius fridge to stop maturation, until used for experiments. 5 tubes were kept in the incubator until the males hatched, to have walking males for assay. To obtain mixed broods, multiple females were placed into a test tube, one male was added and the test tube was placed into the incubator for approximately one day. After that one day the mated females are each placed into a test tube containing 3 host pupa of the *Calliphora* species. The same procedure as the all male broods was followed to reach "black stage" *Nasonia* pupae.

In the following paragraphs the methods of the research will be explained per research question.

Do male *N. vitripennis* of the strain ASYMCX show a significant preference for parasitized fly pupa containing females.

An arena setup was used, the arena is a petri dish divided into 8 parts and a middle circle. These petri dishes were used only once and discarded after every experiment. In each of the 8 parts of the arena a host is placed. Depending on the experiment the specifics of the hosts differ. The hosts have been taken out of the refrigerator an hour in advance to warm up to room temperature. After placing the hosts in the arena a virgin male is placed in the middle. The virgin male was observed for the duration of 4 minutes. The time spent in contact with the host is timed with a stopwatch and documented into an excel sheet. This test is repeated 26 times to obtain a suitable sample size. To take away directional bias 2 identical arenas were created. One arena was used in its normal right side up way, and the second arena was placed 45 degrees off axis. In both arena's 13 repetitions were done.

To obtain an answer for the research question, two separate tests were done. Firstly there was a check for virgin males having a preference for parasitized or unparasitized pupae. The arena was used and alternatively parasitized (P) an unparasitized (U) host were placed in the arena, figure 1a shows the arena setup used.

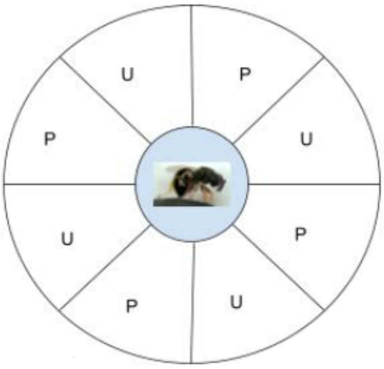

Figure 1a

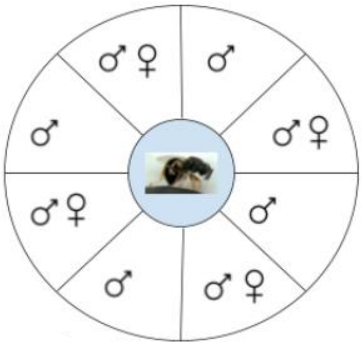

Figure 1b

For the second test the same arena setup was used but now the hosts were either parasitized by a virgin female rearing an all male brood, or parasitized by an mated female rearing a brood containing males and females (mixed). To be sure of the female being fertilized, one of the pupa in each test tube was cracked open and checked for the presence of females. Figure 1b shows the arena setup used.

Do male *N. vitripennis* show a significant preference for pupae containing a higher proportion of females.

For this experiment the pupae that were used in the experiment mentioned above were used again. During the previous experiment the pupae were collected and filtered into “touched” and “untouched”. With touched meaning the walking male had contact with the pupae, and untouched meaning the walking male had not been in contact with the pupa. All the pupa containing females are cracked open and the amount of females is documented in an excel spreadsheet. With this spreadsheet the proportions were calculated and used in the results.

Do male *N. Vitripennis* have a significant preference for pupa containing conspecific larvae or the larvae of other species.

The four species were all reared under the same circumstances. To check if parasitation was successful one of the pupa in the test tubes was cracked open and checked for *Nasonia* pupae. For the assay an arena setup was used with 4 choices. In figure 1c the setup is shown. The arenas were discarded after every trial. In the middle of the arena a virgin *N.vitripennis* male was dropped. The test ran for 8 minutes. Every species had its own timer and this was started every time the virgin male was in contact with a pupae from that species. The cumulative times were noted down in an excel spreadsheet. The experiment was ran with n=25 sample size.

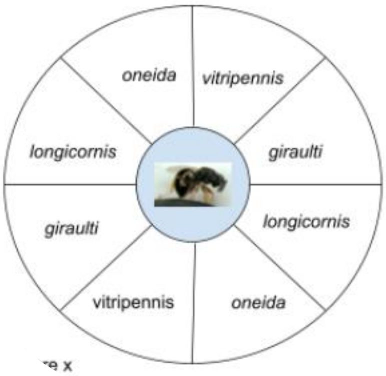

Figure 1c

Is the genetic location for parasitation detection on the same locus as peach/white eyes?
BKBWG mixed broods were made by mating long winged white eyed males, with peach eyed females. After the broods reached black stage they were placed into the fridge to await assay. Multiple pupae were cracked open, and the males were separated into peach and white eyed individuals. The individuals were sorted by colour and put into two different test tubes. These were placed back into the incubator to create walking males for assay.

To confirm that the white and peach eye does correspond with the specialist and generalist gene, a parasitation control was performed. 10 host pupa were placed into a test tube, this was done for approximately 50 test tubes per eye colour. After four days the females were taken out to control for the lifespan of the wasps. The results were pooled in an excel spreadsheet.

For the experiment the same setups as figure 1a and 1b were used. The unparasitized versus parasitized is done for both the white eyed males and the peach eyed males. With a sample size of 25. The all male brood versus the mixed brood is also done for both the white eyed males and the peach eyed males and also with sample size 25. Results were written down in a table in an excel sheet.

Analysis
After the results have been pooled in excel spreadsheets. Analysis was done using Rstudio. Results were plotted using the "ggplot2" package, checked for normality and afterwards corresponding tests were performed, in all these corresponding tests the sample means were compared to each other, determining if there is a significant difference.

Results:

General observations:

A behavior was consistently seen when the virgin males had made their choice. There was "drumming" of the antenna and rubbing of the hind legs together making the abdomen rise in the process. This behavior was seen when the males were in contact with the pupa. But also when they were in the vicinity of a pupa they later would touch.

In the next section the results will be categorized per research question.

Do male N. vitripennis of the strain ASYMCX show a significant preference for parasitized fly pupa containing females.

When giving the *N. vitripennis* virgin males the choice between parasatised versus unparasatized pupa. Most males roamed around the arena for the first few seconds before choosing a pupa of their liking. Most of the time they would choose one pupa and stay there the rest of the time. With n=26 trials done the virgin males showed a significant preference for the parasitized pupa by spending more time being in contact with them. The same setup was used in the next test where the males were given the choice between all male broods in pupa, or mixed brood in pupa. The males did not show a clear preference for one of the choices. Here the same behavior was observed as before. The males roamed around the arena before choosing a pupa of their liking. With n=19 trials the virgin males did not show a significant preference for either the all male brood or the mixed brood. Significance tests show a significant difference between parasitized versus unparasitized. But an insignificant difference in the male versus mixed brood experiment. We can hereby conclude that in the case of *N.vitripennis* (ASYMCX) the males have a significant preference for parasitized pupae, but an insignificant preference for either mixed or all male broods.

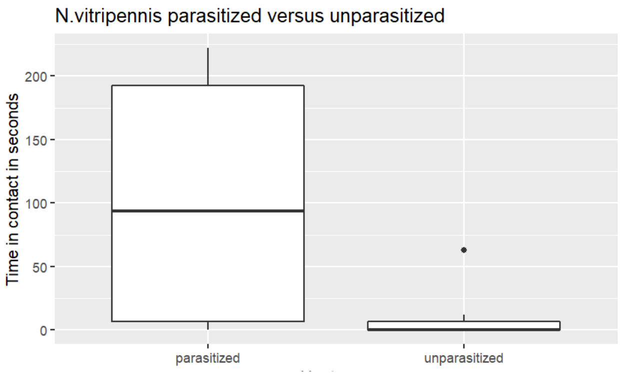

Figure 2a

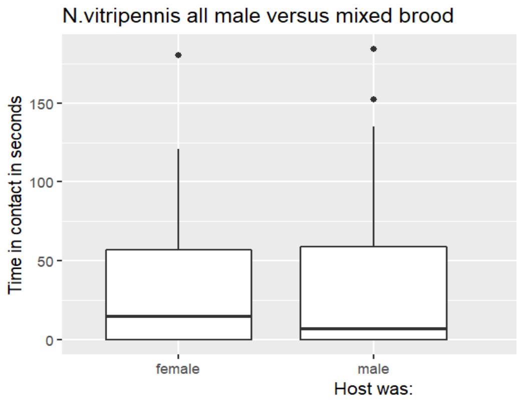

Figure 2b

Figure 2a shows a significant difference in time spent on the host between parasitized and unparasitized hosts. The data was checked for normality via a shapiro-wilk test: p= 2,692e -9. Rejecting the hypothesis that the data is normally distributed. The significance in difference of the mean was measured by a wilcoxon signed rank test: p= 2,315e-5. Giving the result that the difference in means is significant. Figure 2b shows an insignificant difference between means in time spend on all male brood host or mixed brood hosts. The data was checked for normality using a shapiro-wilk test giving: p= 3,268e-6 Rejecting the hypothesis that the data was normally distributed. A Wilcoxon signed rank test gave p= 0,8581. P>0,05 taking the hypothesis that the two means do not differ significantly.

Can male N. vitripennis sense the amount/proportion of females in a parasitzed host pupa.

The all male and female broods that were touched during the experiment rearing results for figure 1b were marked and put aside. From this the following statistics were obtained. There were a total of 148 pupa used in the experiment of figure 2b. Figure 3a gives insight into how the distribution of touched, untouched, all male and mixed broods is. The pupa were subsequently cracked open and the number of females and males inside was counted. This was done using a microscope. The proportions of females inside the host was calculated by:

$$Proportion\ females\ inside\ =\ number\ of\ females/\ total\ pupae\ inside$$

The proportions that came out of the mixed broods is depicted in figure 3b. The statistical tests show a non significant difference in the means of touched and untouched. We can hereby conclude that *N.vitripennis* do not show a significant preference for hosts with a higher amount of females inside.

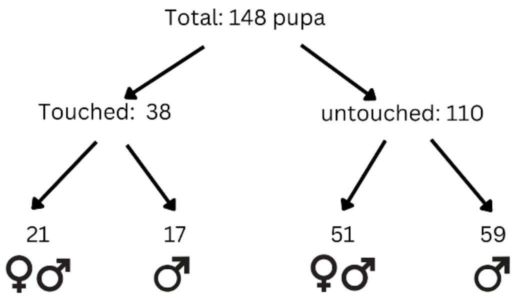

Figure 3a

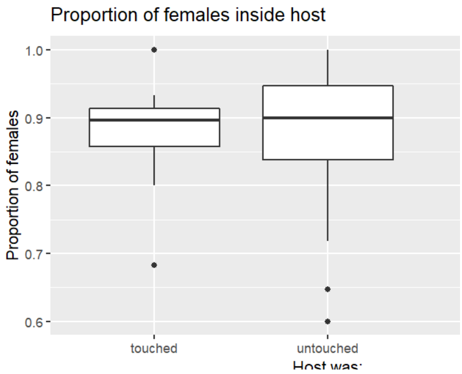

Figure 3b

Figure 3a shows the distribution of how the pupa were touched during the experiment from 1b. Figure 3b shows the proportion of females within the touched hosts that were both mixed broods. The data was tested for normality and with p=0,0003283, It was found that the data was non normal. Comparing if the two means significantly differ a wilcoxon signed rank test was used. This test reared p= 0,5593. This means that we do not reject the null hypothesis and the means do not differ significantly.

Do male *N. Vitripennis* have a significant preference for pupa containing conspecific larvae or the larvae of other species.

The four *Nasonia* species were all reared in the same conditions. *N. Giraulti* failed to parasitize or were stuck in diapause and thus unusable for the experiment. This is why it was decided to alter the experiment in order to be able to run it at a decent sample size (n=27). The alterations to the experiment are:

- As the sample size of *N. giraulti* is 0, this species was excluded from the experiment, continuing the experiment with the remaining three species.
- As the “choice” for the virgin male *N.vitripennis* was cut by ¼ the total initial time was also cut by a fourth. Rendering a 6 minute trial.

Not only *N.geraulti* had a low sample size. *N. Longicornis* was also low, with approximately 20 usable pupae. This is why it was decided that the pupa were going to be reused. After using them the first time they were stored in a large test tube. When no fresh pupa remained, a random pupa out of the tube was picked to use for assay, shaking the large test tube in between picks. For this experiment the results are depicted in figure 4:

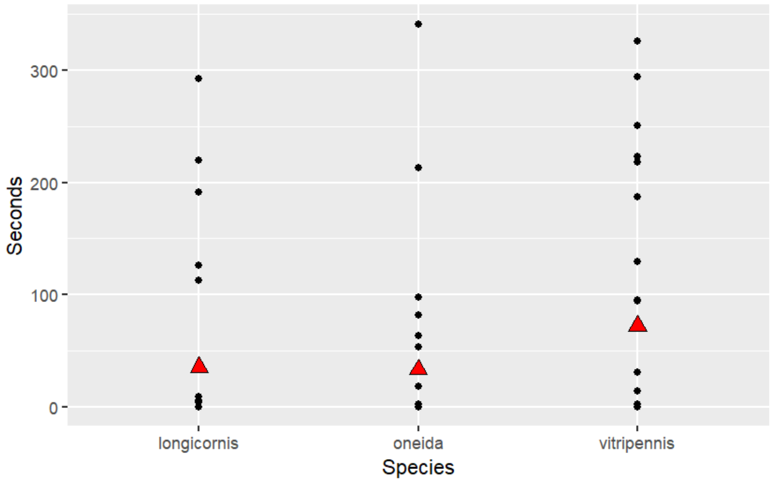

Figure 4

Figure 4 shows the data collected in the Interspecies experiment. The red triangles are the means per data column. The data was checked for normality doing a shapiro test, and it was found that p= 1,69e-13. This means that the hypothesis that the data is normally distributed is rejected. Even though the data was not normal an one sided anova test was chosen. The anova test showed a p=0,1976 meaning that the null hypothesis will not be rejected and the means are not significantly different. Double checking this with the emmeans package in Rstudio and pairs(), all of the combinations were indeed P>0,05. So no mean differs significantly from each other.

Is the genetic location for parasitation detection on the same locus as peach/white eyes?

The experiment was started by separating the all male broods with walking males in peach eyed individuals and white eyed individuals. This is where it was discovered that there was a discrepancy between the number of males for the two colors. For every white eyed individual that was found, 10 peach eyed individuals were found. In the end this caused the decision to delay the experiment 2 weeks to be able to host and hatch more walking virgin males. With the males available a parasitation experiment was done. Both the peach and white eyed females were taken out of the test tubes after a set amount of time to factor out the lifespan.

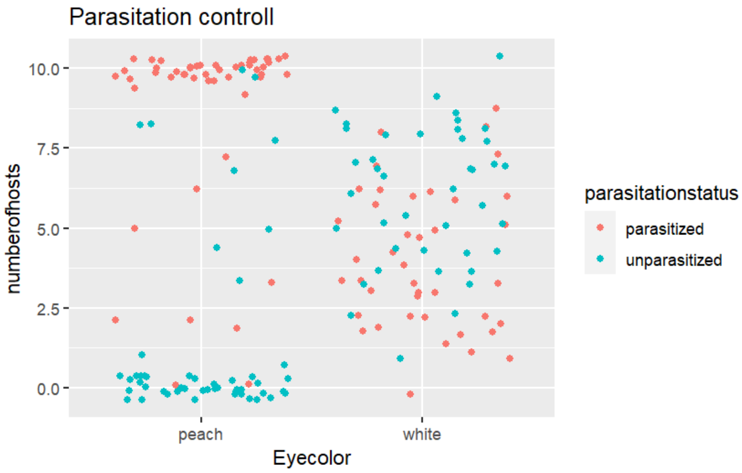

Figure 5

In figure 5, it is shown that peach eyed individuals had no problems parasitizing and in general parasitized all of the hosts that were in the test tube. This confirms its generalist background. It also shows that the white eyed individuals parasitized less, also confirming the theory where they have the specialist genes. This parasitisation experiment thus proves that the peach eyed individuals have the generalist gene and the white eyed individuals have the specialist genes.

Two weeks later the experiment was relaunched, using the “old” males first because the newly hosted males were not ready. Of these old males only the peach eyed remained as the white eyed males had died. Preliminary results on the old males showed a lot of disinterest in the pupa. And in general resulting in a lot of zero values (no hosts being touched). After the males were out and walking the experiment was continued to obtain an ample sample size. It was found that with these younger males the interest was far bigger and the amount of zero samples shrank. The findings of the parasitized versus unparasitized are found below in figure 6

Because this effect was seen in the parasitized versus unparasitized, it was decided that also with the all male versus mixed brood a small sample size of younger males should be redone. This to see if there is an effect here too. In the graphs below the data obtained is depicted. The results show a significant difference for the white eyed individuals. Just by the looks and behavior of the young and old individuals a direct difference was observed. The younger individuals showed more interest in the pupae and walked around more, whereas the older individuals tend to stand in one place. Statistical tests disproved these observations. The means of young and old peach eyed individuals being insignificantly different (p=0,2023). Furthermore it was found that there was no significant difference in means with the all male or mixed brood, concluding that the males do not have a preference, this is depicted in figure 7.

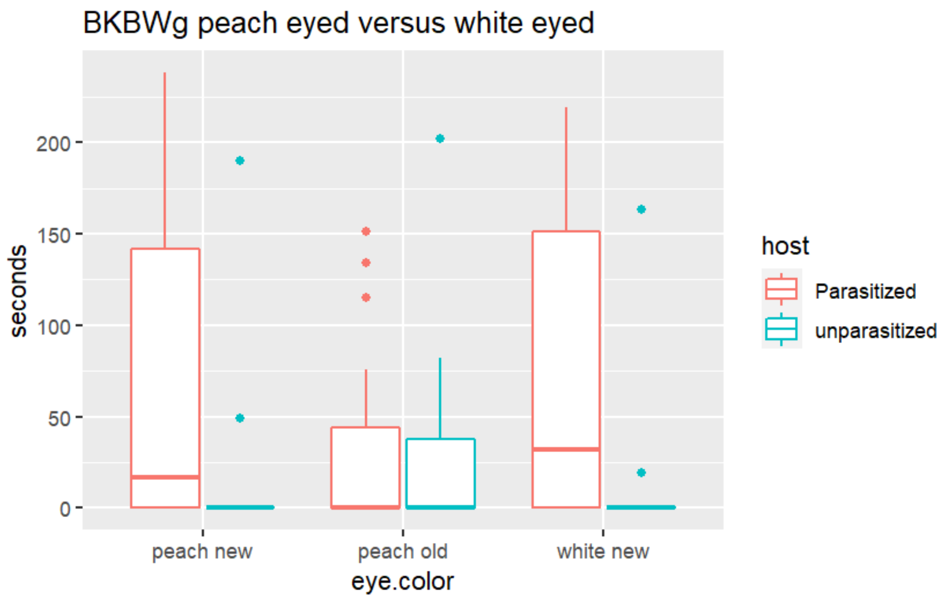

Figure 6

Figure 6 shows the data obtained from the experiment. The data is split out into the peach eyed and white eyed individuals. And within the peach eyed individuals the distinction between the young and older males is made. As can be seen the difference in mean is ther with the new peach males and the new white eyed males but not in the older peach eyed individuals. The data was first tested for normality using a shapiro test. This test gave: p= 2,167e-15, rejecting the null hypothesis that the data is normally distributed. The tests done to determine whether the means of the unparasitized versus parasitized samples were the same is:

- Young peach eyed individuals: a wilcoxin signed rank test gives p=0,1692, therefore not rejecting the null hypothesis that is no difference in means. Meaning that there is no significant difference between the means. But, the sample size in this is very low (n=6), in comparison to the other 2 factors(n=20, n=24). This means that the statistical power of this test is far lower than the other 2. This increases the likelihood of a type 2 error (false negative conclusion).
- Old peach eyed individuals: a wilcoxon signed rank test gives p=0,8537, therefore not rejecting the null hypothesis that is no difference in means. Meaning that there is no significant difference between the means.
- Young white eyed individuals: a wilcoxon signed rank test gives p= 0,001014, therefore rejecting the null hypothesis that there is no difference in means. Meaning that there is a significant difference in means.

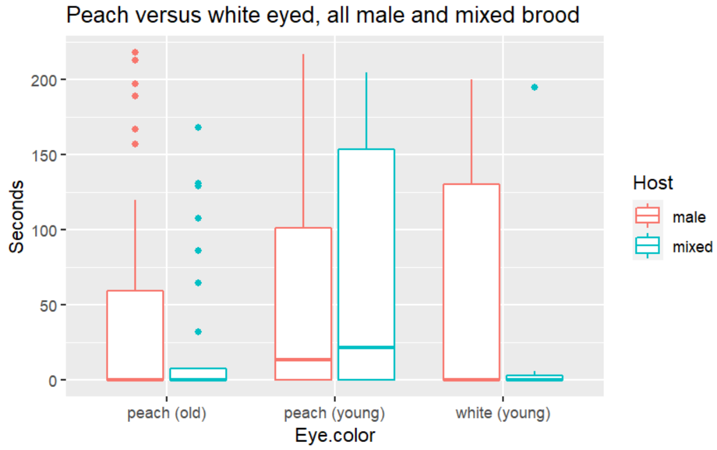

Figure 7

Figure 7 shows the data obtained from the peach and white eyed assay for all male and mixed broods. Within the peach and white eyed individuals the distinction between young and old walking males is made. The data was checked for normality and with p= 1,503e-13 The data was not normally distributed. The means of the four different observations were compared using a wilcoxon signed rank test.

- Old peach individuals: a wilcoxon signed rank test gives p=0,4534, therefore not rejecting the null hypothesis that is no difference in means. Meaning that there is no significant difference between the means.
- Young peach individuals: a wilcoxon signed rank test gives p=0,9054, therefore not rejecting the null hypothesis that is no difference in means. Meaning that there is no significant difference between the means.
- Young white individuals: a wilcoxon signed rank test gives p=0,5514, therefore not rejecting the null hypothesis that is no difference in means. Meaning that there is no significant difference between the means.

Discussion:

The results showed consistent and inconsistent results with research previously done by Prazapati et al.. It was found that male *N.vitripennis* showed a significant preference for the parasitized pupae, but contrary to their work in this research it was found that males could not distinguish between mixed and all male brood. Furthermore it showed that when the male "chooses" a mixed brood pupae it does it at random rather than maximizing the proportion of females inside. For the interspecies host specifity it did not show any significant results for its own or other species. For the BKBWG strain the results were all insignificant except for the white eyed males having a significant preference for parasitized pupae.

During the experiments and midway presentations multiple questions were raised, things that could elaborate on this experiment or things that could have been controlled for that have not been controlled at this point in time.

A problem that was encountered early on in the experiment was the difficulty of parasitisation of the specialist species: *N. longicornis, N. oneida* and in special *N. giraulti.* Not only *did N. gerault*i have a low parasition rate, but when checking almost half of the larvae had gone into diapause rendering them useless for our experiment. In the end this led to a sample size so low that the interspecies test had to be run without *N. giraulti* being present. The hosting could be done by mass hosting to have a greater amount of parasitized hosts.

In general the low parasitation caused a difference in sample size, in the perfect scenario every test would have been run with the same amount of samples (n=26). But in almost all of the experiments done this sample size has been reduced. With a smaller sample size the effect of outliers is bigger and it decreases the statistical power. The decrease in statistical power is a problem as this increases the chance of type 2 errors.

It is unsure if during the parasitized versus unparasitized for *N. vitripennis* the significance is caused by the males sensing the presence of young individuals of their own species or the fact that it was touched by a female. The female was still present (dead) in the test tubes when the parasitized pupae were taken out to be used for assay. When doing further research the pupa that were in the test tube with the female could be treated to get rid of the smell of the female. Or the unparasitized hosts could be in the presence of a female a short interval before the experiment. By doing this the female can be in contact with the pupae but only lay eggs. So no larvae will form in that time.

In the research done previously by Prazapati et al., and the research done in this paper an arena setup is used. In previous research an arena with 6 choices was used. And with these experiments an arena varying from 6 to 8 choices. It is not known if the wasps are overwhelmed by the amount of pupae and are not making the right decision because there are too many pieces to choose from. Or if the arena setup with alternating choices has an effect itself. It could be useful to know if the choices would be different when a different amount of hosts is being used. And testing with random setups. Not alternating the choices but rendering a random distribution of the choices each time, or putting them in one big pile and breaking them open afterwards to check what brood it is.

The behaviour of male *N.vitripennis* on the host and near the host seemed to be the same in some instances. And in other instances the male was observed walking around the pupa but not touching the pupa. For further research the behaviour of the males could be classified. And then a timer could be started from the point where the behaviour was displayed up until the behaviour stops.

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