Investigations of Antennal morphology and Male sperm drop and mating latency using *Nasonia* males

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Certificate of Examination

This is to certify that the dissertation titled "**Investigations of Antennal morphology and Male sperm drop and mating latency using *Nasonia* males**" submitted by **Ms. Sujata (Reg. No. MS15188)** for the partial fulfilment of BS-MS dual degree programme of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

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Dated: June 15, 2020

Declaration

The work presented in this dissertation has been carried out by me under the guidance of **Dr. Rhitoban Ray Choudhury** at the Indian Institute of Science Education and Research Mohali.
This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

Sujata
(Candidate)

Dated: June 15, 2020

In my capacity as the supervisor of the candidate's project work, I certify that the above statements by the candidate are true to the best of my knowledge.

Dr. Rhitoban Ray Choudhury
(Supervisor)

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Abstract

Antennae being an important organ for the insects is studied enormously to see the structures and receptors present on it to help the insect in judging its environment. It also plays a role in finding food and mates. So, it is important to study the changes and effects of these changes in this organ. The first part of this study investigates the antennal morphology of the *Nasonia* males, showing that the main variation was in the flagellum of the antennae. We found that the flagellum of *Nasonia oneida* is the longest among all. This longer phenotype of the male flagella is thought to have advantage of having more sensilla present on it and hence, a better sensing capability for the location of food, nest and mate.

The males of short-lived parasitoids are thought to be born with a lifelong sperm stock causing the male to lose its potency after multiple mating. In the second part of this study, our investigation of the mating latency in males showed variations in the mating capability among the four species of *Nasonia*. The males of *Nasonia vitripennis* and *Nasonia giraulti* showed a higher rate of mating as compared to *Nasonia longicornis*. However, *Nasonia longicornis* showed the highest rate of successful inseminations while *Nasonia vitripennis* showed the least.

CHAPTER 1: To study the antennal length variation in the *Nasonia* species

1.1 INTRODUCTION

Antennae are very important organs present in the head region of most of the insects. The antennae of an insect help in various functions which differ amongst species. Some of the common examples among which are: smell, taste, touch, thermal and humidity senses, communication, detection of mate pheromones, nesting places, migratory routes, orientation during flight and measure the flight speed etc. (Polidori C, Nieves-Aldrey JL; 2014).
A typical antenna has three parts: scape, pedicel and flagellum. The scape is the part connected to the head of the insect, pedicel is the short part joining scape and flagellum, and flagellum is the longest part containing sensory organs. The flagellum mainly consists of multiple segments containing receptors for tactile and olfactory senses as well as chemosensory hair (Slifer E. H., 1969).

Figure 1.1: Different types of Antennae

(SOURCE: https://allyouneedisbiology.wordpress.com/tag/wasp-antennae/)

Nasonia is a parasitoid wasp genus which infects the flesh-eating fly pupae and lay their eggs in it. *Nasonia* is haplo- diploid. *i.e*., the fertilized eggs produce females and unfertilized eggs produce males which are diploid and haploid in nature respectively (Whiting 1967). There are four known species of *Nasonia*; *N. vitripennis*, *N. longicornis*, *N. giraulti* and *N. oneida* (Darling and Werren 1990; Raychoudhury *et al*. 2010). The males and females are easily distinguishable on the basis of their body (both length and width) and wing sizes (Girault and Sanders, 1909; Whiting, 1967) as well as ability to fly (only females fly).

Previous studies on antennal morphology of 126 Chalcidoid species of wasps show evidence that the body size and antennae length are found to be closely related showing species with larger body sizes have smaller antennae length (Symonds MRE & Elgar MA, 2013) and vice- versa. Previous studies have investigated the structures present on antennae (Slifer E. H., 1969; Wibel, 1984) in *Nasonia vitripennis* and shape variation among the male antennae of the four species (Raychoudhury *et al.,* 2010).

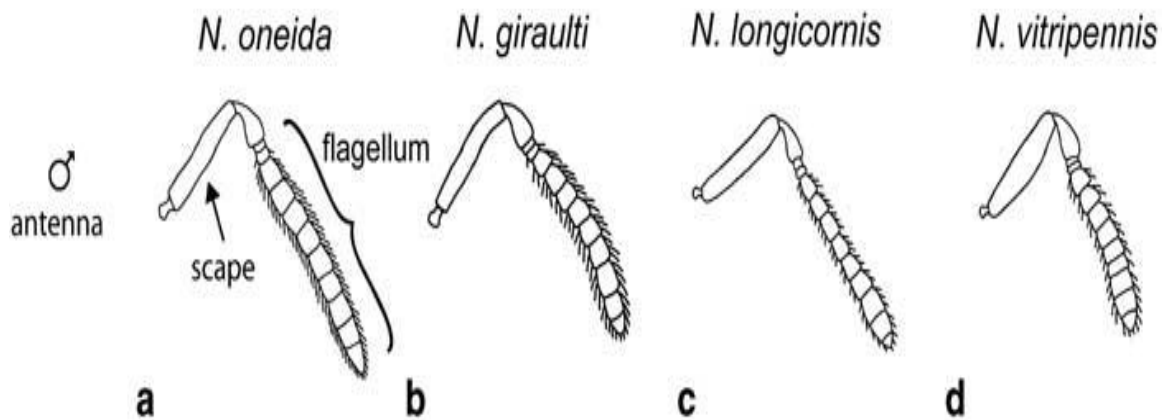


Figure 1.2: Morphological differences in antennae of *Nasonia* species (Raychoudhury *et al*., 2010); angulate scape of *N. oneida* (a) and *N. giraulti* (b), cylindrical scape of *N. longicornis* (c) and spindle-shaped scape of *N. vitripennis* (d). *N. oneida* with narrow male antennal flagellum (a), *N. longicornis, N. giraulti* and *N. vitripennis* with progressively wider antennal flagella, respectively (b, c and d).

However, the *Nasonia* species involved have almost similar body size around 2- 3 mm (Darling and Werren, 1990). This study deals with the investigation of differences in the antennal length of all the four species having similar body size.

1.2 MATERIAL METHODS

1.2.1 LIVE STOCK:

The experiments were done using AsymCx- a cured strain of *Nasonia vitripennis*, Nlmn- a strain of *Nasonia longicornis*, NGRV2- a strain of *Nasonia giraulti*, and NONY-a strain of *Nasonia oneida*.

The *Sarcophaga dux* fly pupae were used as hosts for *Nasonia* species. The adult flies were raised on milk powder with crushed sugar as food and were given chicken liver and blood to lay eggs onto. These eggs were transferred to fresh chicken liver after 24 hours and allowed to grow from larvae to pupae at room temperature for 4 days. On the fourth day, the larvae were transferred to the incubator maintained at 28°C to pupate. The pupae were then kept at 4°C when they reach dark color stage to stop their further development to an adult fly so that they can be used as hosts for *Nasonia* eggs.

The mated females were given fly pupae (4 female- 4 host per vial) for 48 hours, which were then removed and the parasitized pupae were kept at 25°C, 50% humidity and 24 hours- 60% light for the maintaining a continuous culture of the wasp strain. The adult individuals emerge after 14- 16 days of parasitization by the female. Wasps used in the experiments were taken from this culture.

1.2.2 INSTRUMENTS and WARES:

- Plastic vials (17x25 mm)
- Cotton plugs
- Compound microscope
- Scalpel forceps, brush, needle, glass slide, Vaseline, tissue and 70% ethanol

1.2.3 EXPERIMENTAL DESIGN:

1. The virgin females were collected on the 12th day from mated female hosting and hosted as a setup with (2 females- 2 hosts) in five vials for each species.
2. These virgin hosting were kept in the incubator at 25°C for the females to lay egg and the further development of these eggs to adult males for 14- 16 days (depending on the species).
3. After the emergence, the host pupae were removed and the freshly emerged males were collected in fresh vials.
4. These males were then put in a refrigerator at -40°C for about 5-10 minutes to kill them.
5. These dead males were then put under the microscope one by one to dissect their antennae and head apart from their body with the help of forceps and scalpel.
6. The antennae were straightened using Vaseline, brush and needle and the head was positioned in a way that, inter- ocular region was visible.
7. Images the clicked by the camera (Axiocam 105 color) mounted on the compound microscope (Stemi 305).
8. The length of full antennae, only the flagellum and inter- ocular distance were measured using ZEN 2.3 software (Carl Zeiss Microscopy GmbH, 2011).
9. The images for all the data points were saved and revisited to measure the length of the captured images of antennae of all the four species.
10. Both flagellar length and inter- ocular distance were measured for individual males. The length of both the left and right flagellum of the antenna was measured and normalized by dividing the average value with inter- ocular distance to account for any length variations present due to overall body size differences between males.

1.3 RESULTS

The measurements of the antennae showed a noticeable variation in the average flagellar length among the four species (see figure 1.3).

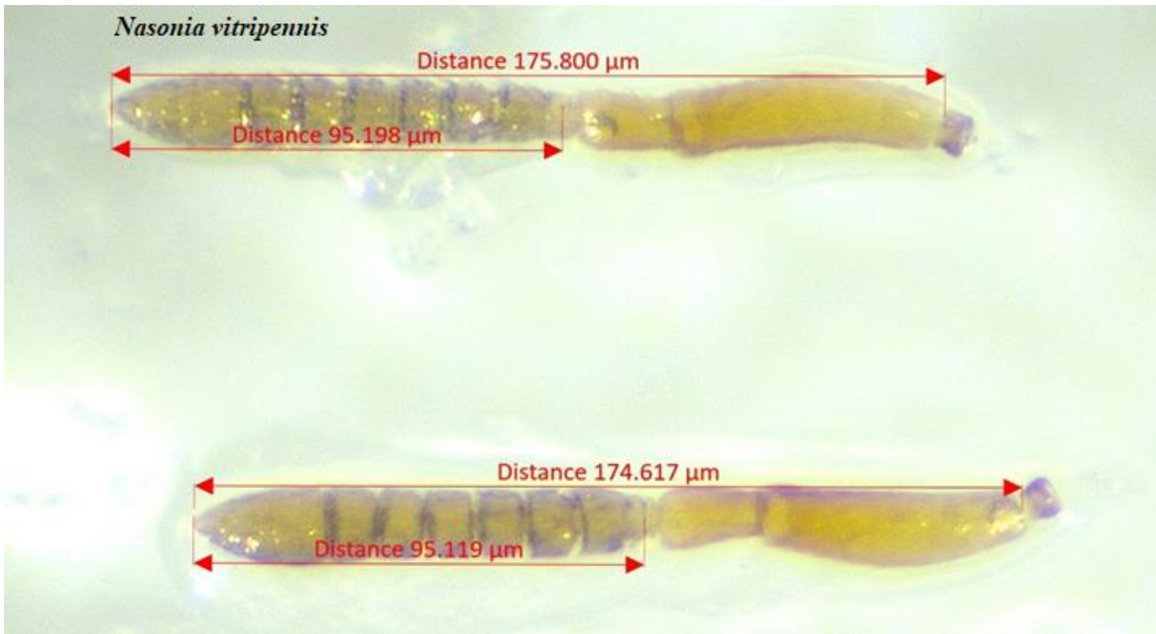
The mean ± standard deviation for average flagella length was calculated for all species and was found to be 95.76 ± 3.38 µm for *N. vitripennis* (N=15), 93.08 ± 3.11 µm for *N. longicornis* (N=15), 82.97 ± 6.32 µm for *N. giraulti* (N=15), and 108.53 ± 3.58 µm for *N. oneida* (N=15).

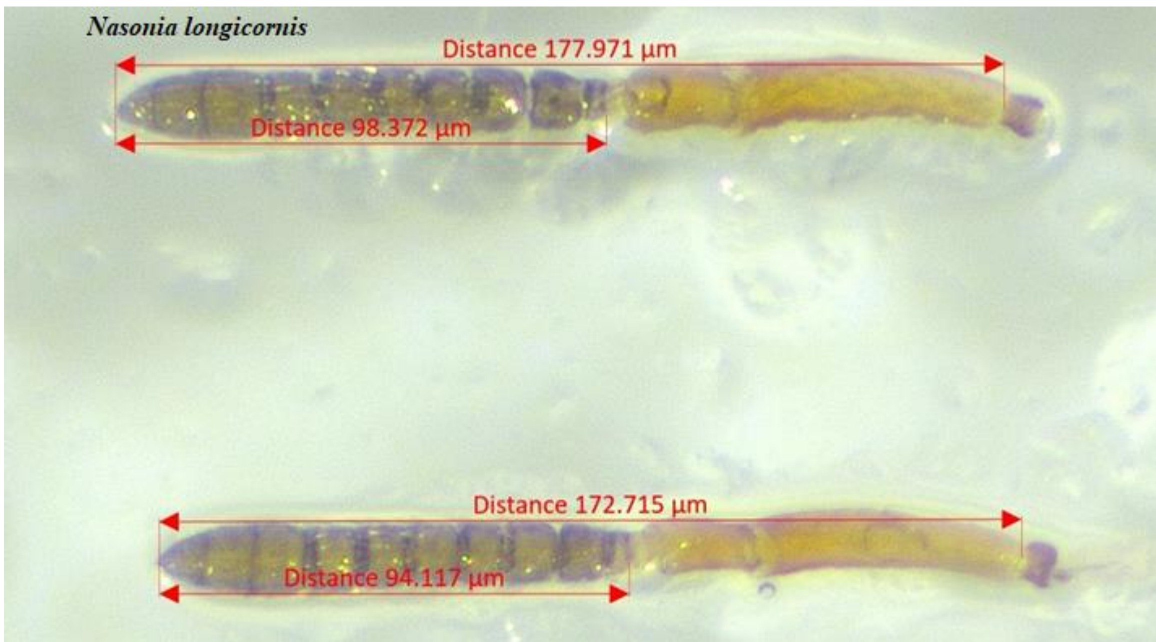
Similarly, for inter- ocular distance, the values of mean ± standard deviation was found to be 84.06 ± 4.06 µm for *N. vitripennis* (N=15), 78.32 ± 3.35 µm for *N. longicornis* (N=15), 77.54 ± 5.96 µm for *N. giraulti* (N=15), and 82.3 ± 3.2 µm for *N. oneida* (N=15).

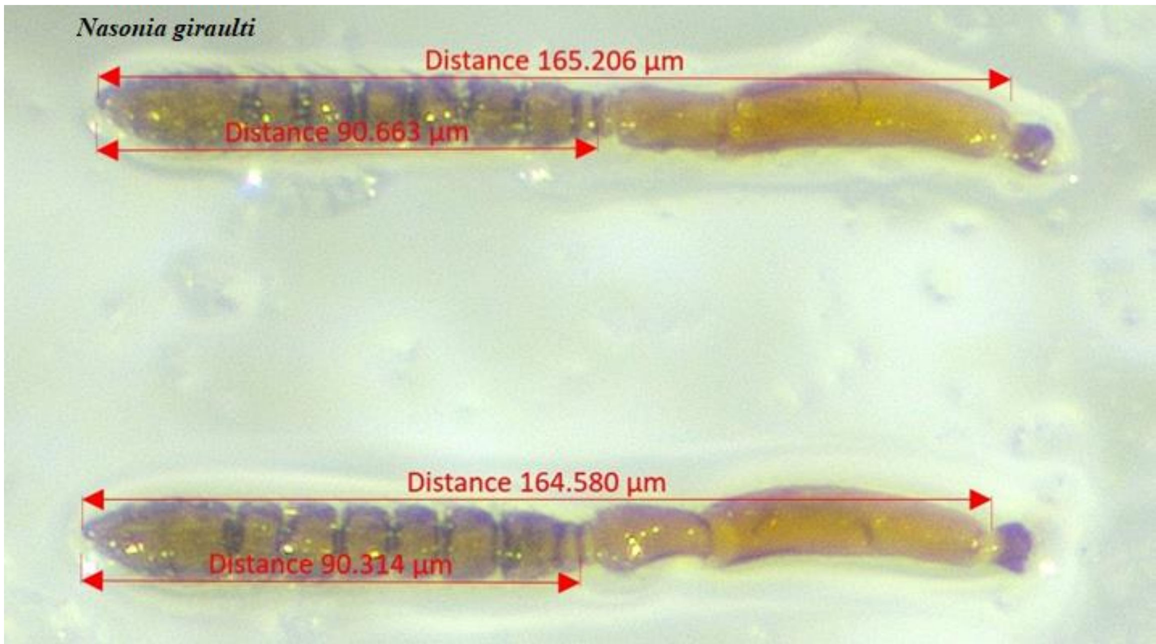
Additionally, the mean ± standard deviation for the flagellum length (FL) to inter- ocular distance (IOD) ratio were found to be 1.14 ± 0.03 for *N. vitripennis* (N=15), 1.19 ± 0.027 for *N. longicornis* (N=15), 1.07 ± 0.028 for *N. giraulti* (N=15), and 1.32 ± 0.034 for *N. oneida* (N=15) (see table 1.1).

From the above data, *N. oneida* has the highest flagellar length and FL/IOD ratio among all the four species, while *N. giraulti* has the lowest. However, *N. vitripennis* and *N. longicornis* has somewhat similar mean values for flagellum length and FL/IOD ratio.

The average flagella length to inter- ocular distance ratio differs significantly ($p < 0.001$) (Mann- Whitney U- test) for all the four *Nasonia* species as shown in table 1.1.


Nasonia vitripennis
Distance 175.800 µm
Distance 95.198 µm
Distance 174.617 µm
Distance 95.119 µm


Nasonia longicornis
Distance 177.971 µm
Distance 98.372 µm
Distance 172.715 µm
Distance 94.117 µm



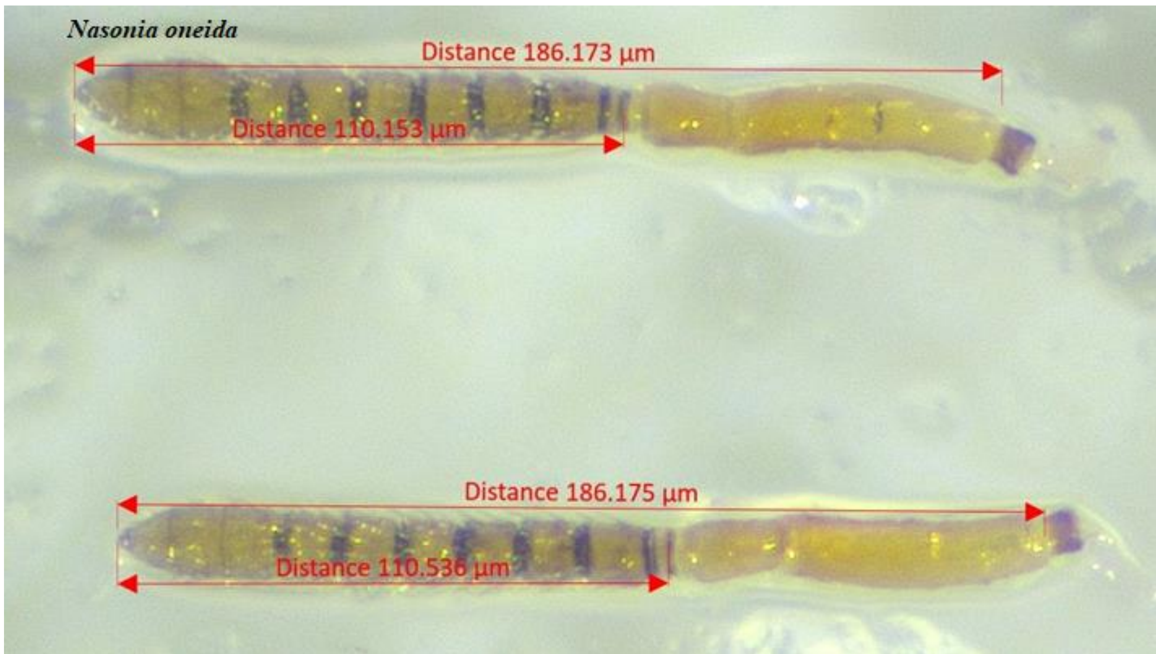


Figure 1.3: Antennae of all the four *Nasonia* species

Flagellum length variation in the four species

Table 1.1: Ratio of mean flagella length with inter- ocular distance in *Nasonia* species

S.No.	Species name	Flagella length (mean of left and right) in µm	Inter- ocular Distance (in µm)	Ratio (Flagella length/Inter- ocular Distance)
1.	*Nasonia vitripennis* (N=15)	95.764	84.066	1.14
2.	*Nasonia longicornis* (N=15)	93.081	78.323	1.19
3.	*Nasonia giraulti* (N=15)	82.975	77.544	1.07
4.	*Nasonia oneida* (N=15)	108.530	82.302	1.32

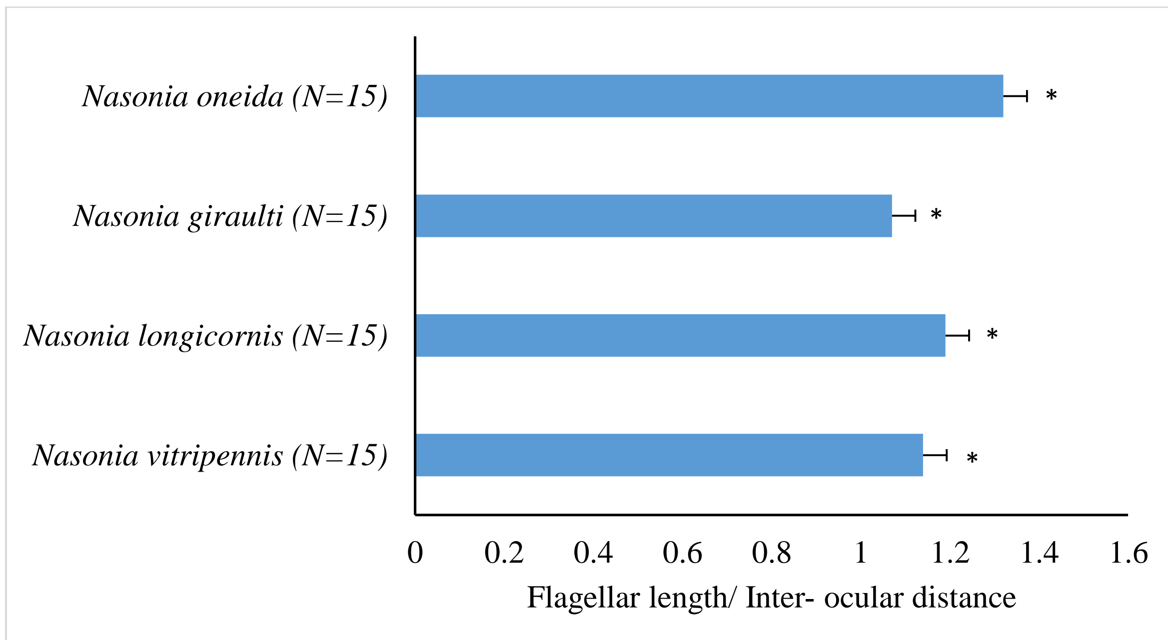


Figure 1.4: Differences in flagellum lengths of the four *Nasonia* species with $p < 0.001$ for all between species comparisons (significant at $p<0.05$ according to Mann- Whitney U- test).

1.4 DISCUSSION

The flagellar length for all the four species differs significantly among each other, with *Nasonia oneida* having the longest flagellum among all ($p<0.001$). The species *Nasonia oneida* was first discovered in Brewerton, New York, USA in 2005 from the blowfly pupae parasitizing the birds. Previous studies showed that males and females of *N. oneida* differ from *N. vitripennis* and *N. longicornis* in their antennal structure with *N. oneida* having an angulate antennal scape (see figure 1.2 (a)) which is similar to *N. giraulti* (see figure 1.2 (b)), *N. longicornis* having a cylindrical scape (see figure 1.2 (c)) and *N. vitripennis* with a spindle-shaped scape (see figure 1.2 (d)). Primarily, *N. oneida* males can be easily distinguished from *N. vitripennis* as they have broad and rounded forewings, while *N. vitripennis* has small forewings. *N. oneida* males can also be distinguished due to a relatively slender antennal flagellum (>9.5X as long as wide), whereas *N. giraulti* and *N. vitripennis* male flagellum are shorter and wider (<8.8X and <8.5X as long as wide, respectively). *N. longicornis* male’s intermediate flagellum is however difficult to distinguish from the other species (see figure 1.2) (Raychoudhury *et al.* 2010).

It has been known previously (Slifer EH., 1969; Wibel, 1984) in *N. vitripennis* that the flagellum has five different sensory receptors present on it. The variation in flagella are common in insect species and larger antennae are said to support better senses by the organism due to presence of more sensilla (Elgar *et al*., 2018). Hence, this longer flagellum phenotype can be assumed to be an adaptation of the organism for better sensing ability due to increased number of sensilla which can in turn help in sensing mates and food availability, better navigation, sensing environment, finding nesting area etc.

This phenotypic observation is *N. oneida* opens up the opportunity to produce genetic introgression study between species (longer flagella -*N. oneida* and shortest flagella - *N. giraulti*) to figure out the genes responsible for the longer flagellum phenotype.

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CHAPTER 2: To investigate the mating latency and sperm drop in *Nasonia* males

2.1 INTRODUCTION

In *Nasonia,* the sex ratio is usually female- biased and under the control of the parasitizing female. This is because females store sperms in their spermatheca after mating and are capable of controlling the release of sperms for fertilization of eggs during oviposition (Werren, 1980; Steven H Orzack and E. D. Parker, 1986).

However, there are studies that showed that males also influence the sex- ratio of the progeny because of different fertilization abilities (producing sperm that were unable to successfully fertilize eggs), incompatibility of paternal and maternal genomes leading to the embryonic death of daughters and active attempt to influence sex allocation as daughters are the ones to carry males genes to the next generation (Hawkes P. D., 1992).

The male's fitness is based on its age (longevity), the number of females it mates with, the quantity and quality of the sperms it produces throughout its lifetime and the sperm allocation among multiple mates (Arnqvist and Nilsson, 2000; Roitberg et al., 2001; Chevrier and Bressac, 2002; Damiens and Boivin, 2005). The adult males of *Nasonia* species have a life- long sperm stock with them as soon as they become adults and are thought to produce no more spermatozoa in the rest of their lifetime, perhaps due to their short (2-4 days) lifespan (M. Chirault *et al.*, 2016). There have been studies done to show that spermatogenesis is synchronous in *Nasonia vitripennis* males and they can allocate their sperm during multiple female matings according to female quality and availability of males for competition (M. Chirault *et al.*, 2016). However, there is no concrete study reported so far about whether the males can replenish their sperms or not.

The focus of this study is to see whether *Nasonia* males have life- long stock of sperms, which they exhaust when mated with numerous females or they replenish their sperms after having exhausted their stock due to consecutive female matings.

2.2 MATERIALS and METHODS

2.2.1 LIVE STOCK

The experiments were done using cured strains of *N. vitripennis* (NVAsymCx), *N. longicornis* (Nlmn), N. *giraulti* (NGRV2), and *N. oneida* (NONY). The mated females were given host pupae (4 female- 4 host per vial) and kept at 25°C, 50% humidity and 60% light 24x7. The adult individuals emerge after 14- 16 days (depending on the species) of hosting the female.

2.2.2 INSTUMENTS and WARES

- Plastic vials (17x25 mm)
- Cotton plugs
- Compound microscope
- Scalpel forceps, brush, needle, glass slide, Vaseline, tissue and 70% ethanol

2.2.3 EXPERIMENTAL DESIGN

1. In a vial, 2 mated females were given 2 fly pupae. After 48 hours, the females were removed and pupae allowed to be at 25°C for the further development of *Nasonia* for 12 days until the wasps reach the black pupal stage of development.
2. The virgin female pupae were collected under the compound microscope (LEICA KL300 LED) by cracking open the fly pupae after 12 days (2 days prior adult stage) in case of *N. vitripennis* and *N. longicornis* at black pupal stage and after 13 days in case of *N. giraulti* and *N. oneida* .were allowed to emerge in the incubator at 25°C.
3. The virgin females were also given fly pupae (2 females-2 fly pupae) to get an all-male adult virgin brood.
4. A freshly emerged (virgin) male from the virgin female hosting was taken and given honey in a vial then kept at 25°C for 24 hours.
5. On the first day after being on honey diet, the male was taken and allowed to mate with virgin females (not older than 4 days) given one after the other until it stopped mating.

6. To ensure that the male successfully mated with the given female, the events from male mounting the female for pre- copulatory ritual to female opening the abdomen for copulation, and post-copulatory ritual for were all observed and timed for each mating. The mating observation where all 3 rituals (pre-copulatory, copulatory and post-copulatory) were observed was taken as "successful mating". The observations lacking any 1 or more of the 3 rituals were discarded from the analysis.
7. The time for start and end of each mating was recorded using a stop watch and noted in the notebook. The start being the male mounting the female and the end being the male getting down and moving away from the female.
8. After the male didn't mate with anymore females, it was again given honey and kept at 25°C for another 24 hours.
9. On the next day (second day of mating), the male was allowed to again mate with virgin females (not older than 4 days) given one by one until it stopped mating.
10. For each male mated, every 3rd mated female (both from 1st and 2nd day matings) was hosted (rest were discarded after successful mating) in a vial separately with 2 fly pupae to parasitize and kept at 25°C for 14-16 days (depending on the species) for the progeny to emerge.
11. The emergence from the vials hosted with mated females was checked for the successful insemination by the males. The successful insemination would result in female progeny in the mated female vials hosted during the 1st and 2nd day matings.

2.3 RESULTS

The continuous mating experiments showed that the males of each species differ in their mating potential and also successful inseminating capability.

The mean ± standard deviation was calculated for successful matings observed on the 1st day for each species' males was (N= 12) is 50.67 ± 11.4 - for *N. longicornis* males, 85.6 ± 16.77 for *N. giraulti* males (N=5), 86.4 ± 24.55 for *N. vitripennis* males (N=5), and 68.4 ± 15.04 for

N. oneida males (N=5). Similarly, for the 2nd day mating by the males it was 35.42 ± 9.28 for *N. longicornis* (N= 12), 62.0 ± 27.18 for *N. giraulti* (N=5), 70.6 ± 9.07 for *N. vitripennis* (N=5), and 53.2 ± 31.45 for *N. oneida* (N=5) (see tables 2.1, 2.2, 2.3 and 2.4).

The data for successful inseminations by the males on the 1st day gives the mean ± standard deviation of 47.75 ± 10.43 for *N. longicornis* (N= 12), 57.4 ± 3.29 for *N. giraulti* (N=5), 22 ± 16.02 for *N. vitripennis* (N=5), and 58.6 ± 9.34 for *N. oneida* (N=5). Similarly, for 2nd day it is 23 ± 5.325 for *N. longicornis* (N=12), 11.6 ± 8.96 for *N. giraulti* (N=5), 10 ± 4.24 for *N. vitripennis* (N=5), and 22.6 ± 12.26 for *N. oneida* (N=5) (see tables 2.1, 2.2, 2.3 and 2.4).

The data for successful matings and successful inseminations on the 1st day of continuous mating was plotted for all the species. In case of *N. longicornis* and *N. oneida* the number for matings observed seems to be somewhat same as that of successful inseminations, while variations were seen in case of *N. giraulti* and the number of successful inseminations seems to be very less as compared to the number of matings observed in *N. vitripennis* (see figure 2.1).

Similarly, the data for 2nd day successful matings and successful inseminations observed was plotted. The data showed a significant decrease in the successfully inseminated females in case of *N. longicornis* when the observed matings were more than 35 on both days (see table 2.1 and figure 2.2), while there was a significant decrease in the number of successfully inseminated females and variations in the number of successful matings observed on day 2 in case of *N. giraulti* (see table 2.2 and figure 2.2) and *N. oneida* (see table 2.4 and figure 2.2) when compared to day 1. However, there was a very little change in successful mating as well as successful inseminating ability of the *N. vitripennis* males on day 2 compared to day 1 (see table 2.3 and figure 2.2).

Table 2.1: Data for successful mating and successful insemination of *Nasonia longicornis*:

S. No.	No. of successful matings (Day1)	No. of successful matings (Day 2)	No. of successful inseminations (Day 1)	No. of successful inseminations (Day 2)
1.	63	43	61	16
2.	51	50	49	19
3.	47	43	46	22
4.	70	25	58	16
5.	47	48	46	22
6.	34	21	34	19
7.	70	29	70	28
8.	46	38	43	28
9.	50	34	46	31
10.	43	26	43	22
11.	48	35	40	22
12.	39	33	37	31

Table 2.2: Data for successful mating and successful insemination of *Nasonia giraulti*:

S. No.	No. of successful matings (Day 1)	No. of successful matings (Day 2)	No. of successful inseminations (Day 1)	No. of successful inseminations (Day 2)
1.	100	90	52	19
2.	104	93	58	4
3.	75	47	58	0
4.	64	37	58	19
5.	85	43	61	13

Table 2.3: Data for successful mating and successful insemination of *Nasonia vitripennis*:

S. No.	No. of successful matings (Day 1)	No. of successful matings (Day 2)	No. of successful inseminations (Day 1)	No. of successful inseminations (Day 2)
1.	126	79	4	10
2.	73	57	10	10
3.	61	66	40	4
4.	83	77	19	16
5.	89	74	37	10

Table 2.4: Data for successful mating and successful insemination of *Nasonia oneida*:

S. No.	No. of successful matings (Day1)	No. of successful matings (Day 2)	No. of successful inseminations (Day 1)	No. of successful inseminations (Day 2)
1.	70	65	67	37
2.	67	52	61	13
3.	62	99	58	28
4.	92	17	64	7
5.	51	33	43	28

The number of successful matings on the 1st day differ significantly ($p < 0.05$) according to Mann- Whitney U- test among the pairs *Nasonia longicornis* and *Nasonia giraulti*, *Nasonia longicornis* and *Nasonia vitripennis* as well as *Nasonia longicornis* and *Nasonia oneida*. But there was no significant difference among the pairs *Nasonia giraulti* and *Nasonia vitripennis*, *Nasonia giraulti* and *Nasonia oneida* as well as *Nasonia vitripennis* and *Nasonia oneida* (see figure 2.1).

The total number of successful inseminations on day 1 are similar in *Nasonia longicornis* and *Nasonia giraulti*, *Nasonia longicornis* and *Nasonia oneida* as well as *Nasonia giraulti* and

Nasonia oneida. However, the number of successful inseminations differ significantly ($p < 0.05$) according to Mann- Whitney U- test when compared to *Nasonia vitripennis* (see figure 2.1).

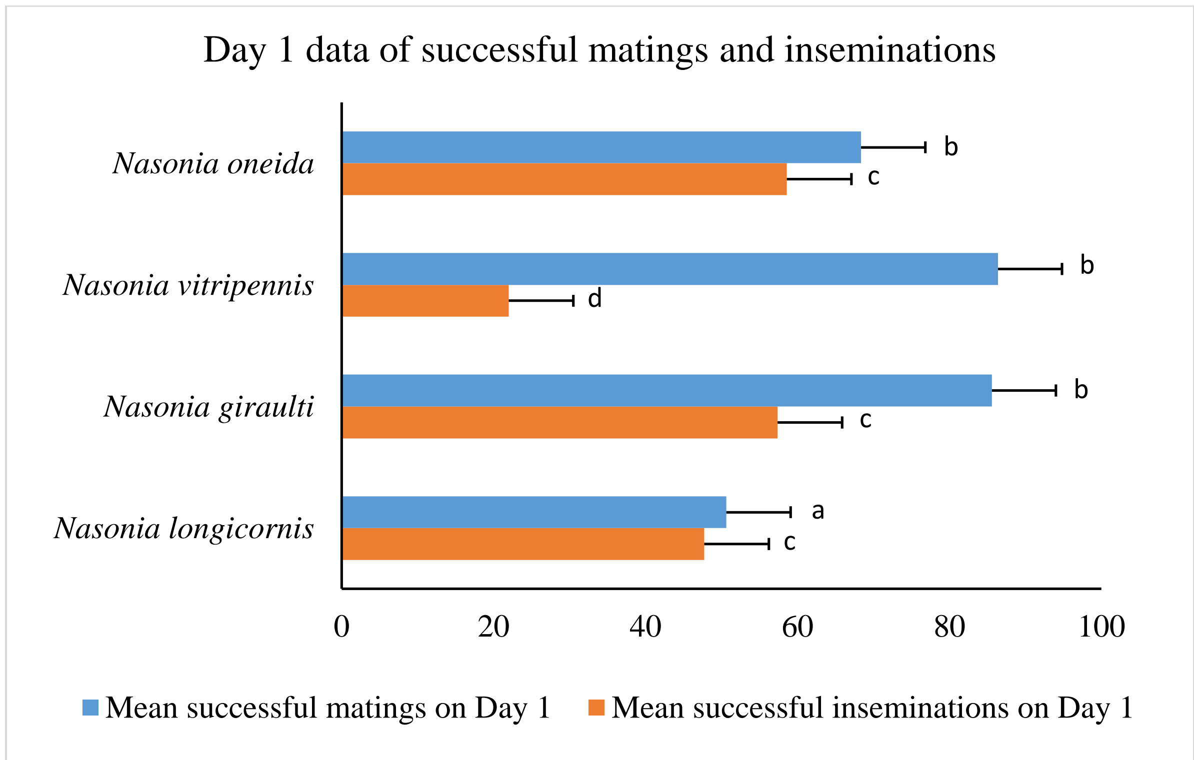

Figure 2.1: Comparison between the species Day 1 mating and insemination data with p-values significant at $p < 0.05$ using Mann- Whitney U- test.

The number of successful matings on the 2nd day differ significantly ($p < 0.05$) according to Mann- Whitney U- test among the pairs *Nasonia longicornis* and *Nasonia giraulti* as well as *Nasonia longicornis* and *Nasonia vitripennis*. However, there was no significant difference among *Nasonia longicornis* and *Nasonia oneida*, *Nasonia giraulti* and *Nasonia vitripennis* or *Nasonia oneida* as well as *Nasonia vitripennis* and *Nasonia oneida* (see figure 2.2).

Also, the total number of successful inseminations on day 2 are similar in case of *Nasonia longicornis* compared to *Nasonia oneida*, *Nasonia giraulti* compared to *Nasonia vitripennis* and *Nasonia oneida* as well as *Nasonia vitripennis* compared to *Nasonia oneida* except

Nasonia longicornis and *Nasonia giraulti* as well as *Nasonia longicornis* and *Nasonia vitripennis* (see figure 2.2).

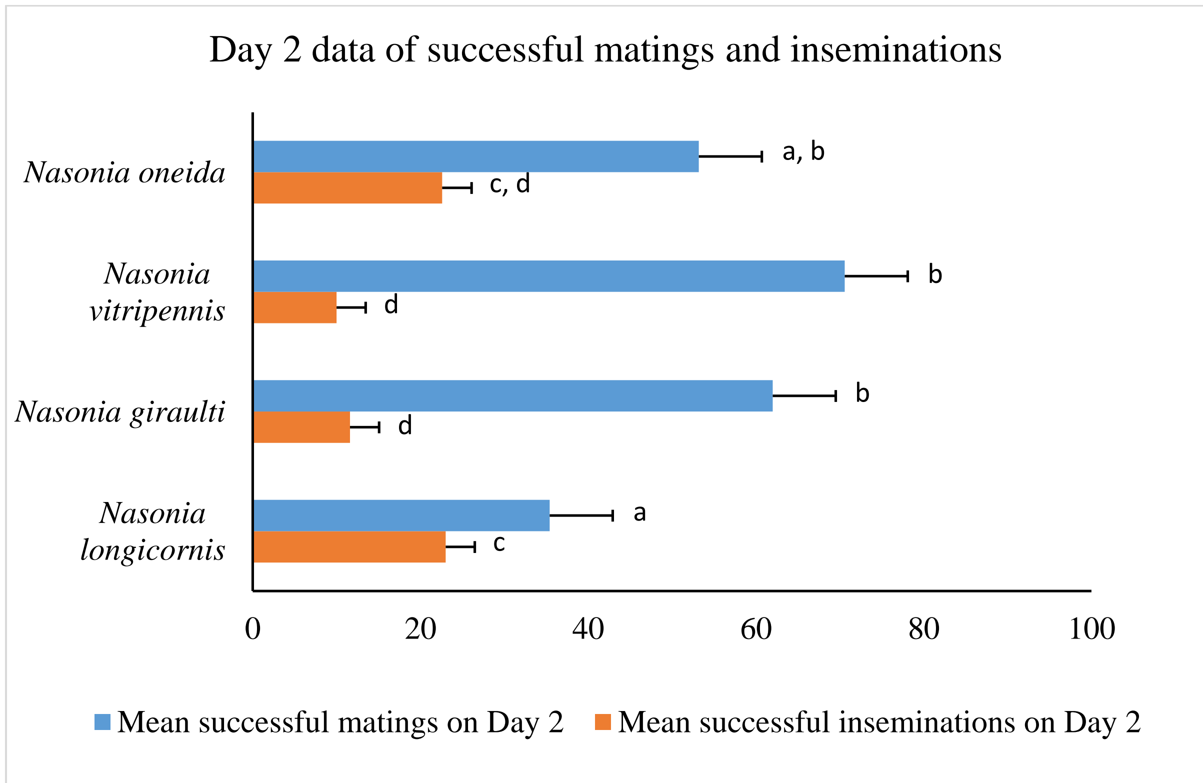

Figure 2.2: Comparison among the species Day 2 mating and insemination data with p-values significant at p< 0.05 using Mann- Whitney U- test.

2.4 DISCUSSION

The males are subjected to sperm depletion after continuous matings. In this study, I observed sperm drop in the males after mating continuously till the male stopped mating with virgin females as there were only males present in the progeny of these mated females. However, the progeny of females mated with the male during second day mating showed presence of females. These observations can be seen as replenishment of sperms during the 24 hours of rest and food. Another reason could be the strategic allocation of sperm by males based on the female size and quality or the late maturation of elongated germ cells present in seminal vesicles of freshly emerged males along with spermatozoa (M. Chirault *et al.*, 2016).

The data we got suggests that the number of successful matings on day 1 as well as day 2 were very low in *N. longicornis* as compared to other species (see tables 2.1, 2.2, 2.3 and 2.4). This suggests that the males of *N. longicornis* might have a poor mating capacity than males of other species. However, the number of successful inseminations in case of *N. longicornis* on day 1 and day 2 were quite high (see figure 2.1 and 2.2). This might be due to the smaller number of successful matings by the males on both days and hence, less depletion of sperm stock.

The males of *N. vitripennis* showed a really low number of successful inseminations on both days as compared to the number of successful matings by them (see figure 2.1 and 2.2). This suggests that the sperm quantity is really low in *N. vitripennis* males than other species. However, it might be a case of low quality and small sized males being chosen for the experiment. Also, the number of data points are not that much to get proper conclusion.

The males of *N. oneida* have mating capability similar to that of *N. giraulti* and *N. vitripennis* on both days but it doesn't differ to *N. longicornis* on day 2 (figure 2.1). However, the successful insemination data showed that *N. oneida* has similar behavior as that of *N. longicornis* and *N. giraulti* on both days but on day 2 it doesn't differ from *N. vitripennis* either (figure 2.2).

The data we got gives us an inference that either the males are consciously retaining their sperm stock as they are consecutively mating with females or the germ cells found in the freshly emerged males get matured with time leading to the replenishment of sperm stock for the second day matings.

Detailed experiments with more data points might give a better insight on whether the sperms are getting replenished actually or is it due to males allocating their sperms accordingly or the late maturation of sperm germ cells.

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