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Marlène Chirault, Christophe Bressac, Marlène Goubault, Charlotte Lécureuil

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1 **Sperm limitation affects sex allocation in a parasitoid wasp *Nasonia vitripennis***

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3 **Marlène Chirault, Christophe Bressac, Marlène Goubault¹ & Charlotte Lécureuil^{1,2}**

4 **Running title:** sex allocation in sperm limited wasps

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6 *Institut de Recherche sur la Biologie de l’Insecte UMR 7261 Université François-Rabelais / CNRS,*

7 *UFR Sciences et Techniques, Parc Grandmont, Tours, France*

8 ¹co-last authors

9 ²Corresponding author. *Email address:* charlotte.lecureuil@univ-tours.fr

Abstract

Insect reproduction is influenced by various external factors including temperature, a well studied constraint. We investigated to what extent different levels of sperm limitation of males exposed to different heat-stresses (34°C & 36°C) affect females' offspring production and sex allocation in *Nasonia vitripennis*. In this haplodiploid parasitoid wasp attacking different species of pest flies, we investigated the effect of the quantity of sperm females received and stored in their spermatheca on their sperm use decisions, hence sex allocation, over successive ovipositions. In particular, we compared the sex allocation of females presenting three levels of sperm limitation (i.e. mated with control, 34°C heat-stressed or 36°C heat-stressed males) on each host they parasitized. To disentangle the potential reduction of sperm quality after a heat stress exposure from that of sperm quantity, we also explored the clutch size and sex-ratio produced by females that were partially sperm limited after copulating with multiply mated males. Independently of their sperm numbers, all types of females produced a similar total number of offspring, but the more limited ones had fewer daughters. Sperm limitation further affected the distribution of daughters' production across time. In addition to constraints acting on female physiology, male fertility should therefore be considered in studies measuring reproductive outputs of insects submitted to heat stresses.

Key-words: Heat stress, multiple mating, sex ratio, sperm use, sperm stock, offspring production dynamic.

29 Introduction

30 In sexual animals, numerous factors such as social, environmental and intrinsic conditions can
 31 affect sex allocation (see Hasselquist & Kempenaers, 2002, Komdeur & Pen, 2002). Sex allocation
 32 theory therefore predicts to what extent and under which conditions organisms should invest into
 33 male vs. female offspring (Charnov, 1982, Hardy, 2002, West, 2009). Hymenopteran parasitoids are
 34 useful models to develop and test this theory (Godfray, 1994, West, 2009). Due to their haplodiploid
 35 reproductive system (i.e. females develop from fertilized eggs while males develop from unfertilized
 36 ones) and their ability to store sperm in their spermatheca after mating, ovipositing mothers of many
 37 species adjust the sex ratio of their offspring by controlling the fertilization of eggs as these pass
 38 through the oviduct (Godfray, 1994, Ratnieks & Keller, 1998, West, 2009). The observation of sex ratios
 39 highly skewed towards females in parasitoid species can especially be explained by the Local Mate
 40 Competition theory (LMC, (Hamilton, 1967)). LMC predicts that in spatially structured populations, in
 41 which matings are likely to occur among kin, a single foundress parasitizing a host patch should
 42 produce only enough sons to mate her daughters, as observed in many parasitoid wasps (Godfray et
 43 al., 1994, Hardy, 2002).

44 Despite the well-documented manipulation of offspring sex ratio by female wasps (Godfray et
 45 al., 1994, Hardy, 2002, West, 2009), one important factor constraining their offspring production and
 46 sex ratio is the quality and quantity of sperm they receive from males (Boivin, 2013, Chevrier & Bressac,
 47 2002, Henter, 2004). In many parasitoid species, males present on average high insemination abilities
 48 (i.e. they have more sperm than required to inseminate their sisters, (Martel et al., 2016), even if
 49 sperm-limited females are observed (Ode et al., 1997, Henter, 2004). Males' capacity to fertilize
 50 offspring varies with age (Damiens et al., 2003), genotype (Khanh et al., 2005) and social status (Kelly
 51 & Jennions, 2011). Successive matings can make parasitoid males even more limited in sperm (Bressac
 52 et al., 2008, Jacob & Boivin, 2004, King, 2000, Steiner et al., 2008). Environmental factors, such as food
 53 quality and availability and chemical and thermal stresses also strongly affect sperm production (e.g.

Blaul & Ruther, 2011, Chihrane & Laugé, 1994, Chirault et al., 2015, Lacoume et al., 2006, Lacoume et al., 2007, Lacoume et al., 2009, Nguyen et al., 2012, Rohmer et al., 2004). These cumulative effects lead males to transfer reduced ejaculates to females, although they continue to attract receptive females for mating (Damien & Boivin, 2005, Assem, 1986). In monandrous species, which represent 80% to 85% of hymenopteran parasitoid species (Gordh & DeBach, 1978, Ridley, 1993), the costs of mating with such males is particularly high, because sperm-limited females are then forced to produce suboptimal sex ratio by laying more sons than predicted by LMC even in good quality hosts (host quality model predicts that males should mainly be laid in poor quality hosts (Charnov, 1982). This raises the question of how females use this limiting resource over the course of their successive ovipositions. Does sperm limitation totally constrain female sex allocation or can female strategically allocate this resource and adjust the sex ratio of their offspring according to their level of sperm limitation? We addressed these questions in *Nasonia vitripennis* (Hymenoptera: Pteromalidae), a gregarious parasitoid attacking pupae of different Dipteran species (Whiting, 1967).

Sex allocation in *N. vitripennis* has been the subject of many studies (Godfray et al., 1994, Shuker et al., 2007, Shuker & West, 2004, Werren, 1980). Females adjust their offspring sex ratio in accordance to the predictions of the Local Mate Competition theory (Hamilton, 1967). Mated *N. vitripennis* females produce strongly female-biased sex-ratio (~88% females) when exploiting a host patch alone (Grillenberger et al., 2008). However, when they mate with non-virgin males, the number of spermatozoa transferred decreases with the number of females a male has previously inseminated (Chirault et al., 2016, Ruther et al., 2009). Females also receive a low number of spermatozoa after mating with males that were exposed to a heat stress during juvenile development (Chirault et al., 2015). Males exposed to high temperature indeed produce a reduced number of spermatozoa throughout their life (Chirault et al., 2015, Chirault et al., 2016). When females are mated with males exposed to 36°C for 24h during the pupal stage, they had about 80% fewer spermatozoa in their spermatheca than those mated with control males (Chirault et al., 2015). Being monandrous (Burton-Chellew et al., 2007), choosing a high quality male is key for *N. vitripennis* female reproductive success

(Ruther et al., 2007). Based on the reduction of male sex pheromones titers, females can discriminate several levels of sperm-limited males after multiple matings and avoid mating with them (Ruther et al., 2009). Nevertheless, females cannot discriminate against heat-stressed males because pheromone titers appear correlated with the number of matings but not directly to sperm limitation (Chirault et al., 2015). Consequently, females inseminated by such heat-stressed males produce a strongly male biased sex ratio of 26% females (Chirault et al., 2015).

In the context of climate change and the increasing risk of animals suffering heat-stress, we investigated to what extent different levels of sperm limitation due to different heat-stresses (34°C & 36°C) affect females' offspring production and sex allocation. Is females' sex-ratio fully constrained by sperm quantity, or can they adjust their sperm use decisions, hence sex allocation, host by host? To test this, we compare the sex allocation of females presenting three levels of sperm limitation (not-limited, partially-limited and very-limited) on each host they parasitized. To disentangle the potential reduction of sperm quality after a heat stress exposure from that of sperm quantity, we also explored the clutch size and sex-ratio produced by females that were partially sperm limited because they mated with multiply mated males.

Material and methods

Insect rearing

Nasonia vitripennis used for this experiment came from the genome reference strain, AsymC (Werren et al., 2010) and were maintained on pupae of *Calliphora sp* at 25°C under constant light and room humidity (Werren & Loehlin, 2009). Under these conditions, males emerged from the host puparium after 13 d, and females after 14 d. Males have reduced wings, do not disperse and mate on the natal patch upon female emergence. In contrast, females are fully winged and disperse after mating in search of new patches of hosts (Grillenberger et al., 2009). This species is monoandrous in nature but in the laboratory could be forced to be polyandrous (Burton-Chellew et al., 2007)

To obtain virgin individuals of both sexes, pupae were collected by opening the host puparium 1 to 4 d before emergence. Each wasp pupa was individually maintained in a 2 mL tube, closed with a cotton ball. Emergences were checked every day and females were used in experiments the day of their emergence. To generate additional males for experiments, virgin females were provided with hosts. The day of their emergence, virgin males issued from such broods were randomly assigned to one of the different treatments (see below for details).

Experimental design

To investigate how *N. vitripennis* females adjust the production and sex ratio of their offspring in relation to the number of sperm stored in their spermatheca, they were mated with either one control male or one sperm-limited male. Males presenting different degrees of sperm limitation were used. They were experimentally produced by submitting them to one heat stress during development, either 34° or 36°C (see below for more details), which are known to differently affect sperm production (Chirault et al. 2015 and this study), or by allowing them to mate 10 times before copulating with the test females, which significantly reduces the ejaculate size at the 11th copulation (Chirault et al. 2016). The average numbers of spermatozoa transferred by these different categories of males during copulation were compared and used to class females into 3 groups (not-limited, partially-limited and very-limited, see Results for details). Data came from previous studies (control and 36°C heat-stressed males: Chirault et al. 2015 ; multiply-mated males: Chirault et al. 2016) and the present study for 34°C heat-stressed males using the same procedure as in Chirault et al. 2015, 2016.

To obtain the different types of males, male pupae were collected from hosts parasitized by only-male broods once they had reached the first pupal stage with uncolored eyes (i.e. 9 days after female oviposition) and they were divided in three groups: male pupae were either (1) maintained continuously in the climate room at 25°C until emergence (for control and multiply-mated males), or

(2) were heat-treated at 34°C for 24h in a climate-controlled incubator then returned to the climate room at 25°C until emergence (for 34°C heat-stressed males), or (3) heat-treated at 36°C for 24h then replaced at 25°C until emergence (for 36°C heat-stressed males). The day following their emergence, males assigned to the multiply-mating treatment were successively provided with 10 newly emerged virgin females: each new female was introduced immediately after the end of the previous mating.

On the day of their emergence, test females were singly mated with either a control male (N=29), a multiply-mated male (N=8), a 34°C heat-stressed male (N=14), or a 36°C heat-stressed male (N=20). All males used were 1 d old, pairs were observed for copulation. After mating, females were transferred to a vial and provided with a cotton ball soaked with a 40% (w/v) sugar solution, as a food source, and 2 unparasitized pupae of *Calliphora sp.*, as oviposition sites. The two hosts were replaced daily during 15 days and stored at 25°C for 9-13 d allowing offspring development until pupal stage. The host puparium was carefully opened to check the presence of offspring; and if so, their number and sex was noted. The two hosts were considered as an oviposition patch, the numbers of offspring developing on both hosts were therefore summed. Although the chosen hosts were at a developmental stage (i.e. at the intermediate phase of the pupal stage, easily recognizable thanks to the brown-red color of their puparium) and a size suitable for female oviposition, some females did not parasitize any of them during one day. Because they did lay eggs on the following days, this temporary absence of oviposition did not reflect a lack of available eggs but perhaps merely a low quality of the proposed hosts. We thus considered the number and sex of the offspring only during days where they actually parasitized at least one host, thereafter called 'oviposition days'. Females that produced no daughter over the complete experiment were considered as virgin and discarded from the data set. Female mortality was also recorded.

Statistical analyses

To define the different categories of females, we compared the number of spermatozoa they stored in their spermatheca according to the type of males they mated with (i.e. either a control male (data coming from Chirault et al., 2015), a multiply-mated male (data coming from Chirault et al., 2016), a 34°C heat-stressed male (data from this study) or a 36°C heat-stressed males (data coming from Chirault et al., 2015)) using an ANOVA test followed by Tukey post-hoc comparisons.

The total numbers of offspring and daughters produced by the different types of females over the course of the experiment were compared using Kruskal-Wallis tests followed by pairwise comparison Dunn's tests with Bonferroni correction, when appropriate. We tested the effect of sperm stored by females on the total number of offspring laid, or sex allocation (number of daughters) for each oviposition days using linear mixed-effect models (function *lmer* from the package *lme4*, family = Gaussian), with the type of males they mated with and the day of oviposition as fixed-effect explanatory variables and female identity as a random-effect term. We checked for homoscedasticity and residual normality. When the interaction term between the *type of males* and *oviposition days* was significant, we further compared the total number of offspring (or daughters) laid by the females mated with the different types of males for each oviposition day performing ANOVAs followed by Tukey post-hoc comparisons.

We further tested the effect of sperm stored by females on their odd sex ratio (in terms of the proportion of daughters produced) for each oviposition day using a mixed-effect logistic regression (function *glmmPQL*) fitted to a quasibinomial error distribution. The type of males they mated with and the day of oviposition were used as fixed-effect explanatory variables and female identity as a random-effect term. When the interaction term between the *type of males* and *oviposition days* was significant, we compared the odd sex ratio produced by the females mated with the different types of males for each oviposition day performing ANOVAs followed by Tukey post-hoc comparisons. Because females inseminated by a multiply mated male did not lay offspring on the 15th oviposition day, we restricted our comparison to the first 14th oviposition days. Finally, we investigated whether female survival over the course of the experiment varied according to type of males they mated with using a

178 survival analysis with a Weibull distribution. All statistical analyses were performed with R 3.1.2 (R Core
179 Development Team, 2013)

Results

Sperm stored by females

The quantity of sperm that females stored in their spermatheca varied with the type of males they had mated with (Table 1). Sperm stock of females mated with a 34°C heat-stressed and a multiply-mated male did not differ significantly and was intermediate compared to the two other types of females (Table 1). We therefore classed them into 3 groups: not-limited, partially-limited and very-limited in spermatozoa. The comparison of offspring production and sex allocation of both types of partially-limited females allowed controlling for a potential effect of heat stress on the quality of the sperm transferred by males.

Offspring production and sex allocation

Over the course of the experiment, females mated with different types of males produced a similar overall number of offspring, but those mated with 36°C heat-stressed males produced significantly fewer daughters (Table 2).

The number of offspring produced per oviposition day varied through time and according to the type of males that mated females (Table 3, Figure 1A). Post-hoc tests showed that the four categories of females presented a similar pattern of offspring production during the first 11 oviposition days: offspring number increased during the first 3 oviposition days, then decreased from the 7th till the 11th oviposition day. Subsequently, offspring production varied with the type of males that inseminated females (Figure 1A). From the 12th oviposition day, a second increase in offspring number was observed for not-limited and partially-limited females, that was more pronounced in females inseminated by multiply-mated and 34°C heat-stressed males. This second increase is delayed for very-limited females.

The number of daughters produced per oviposition day varied through time and according to the type of males that females mated with (Table 3, Figure 1B). Post-hoc tests showed that, from the 3rd day of

oviposition, females inseminated by a 36°C heat-stressed male produced a significantly reduced number of daughters compared to females mated with any other types of males (Figure 1B).

The sex-ratio produced per oviposition day varied through time and according to the type of males that mated females (Table 3, Figure 1C). Post-hoc tests showed that the proportion of daughters produced by females that mated a 36°C heat-stressed male was always lower than that of other females (Figure 1C). The sex-ratio of females mated with a 34°C heat-stressed male started to decrease on the 9th oviposition day and that of females mated with a multiply-mated male on the 12th oviposition day (Figure 1C).

Female survival

Female survival during the experiment did not significantly differ according to the type of males they mated with ($\chi^2 = 3.13$, $df = 3$, $P = 0.37$).

Discussion

The present study revealed that female sex allocation patterns varied with the reproductive quality of their mates in the monandrous parasitoid wasp *N. vitripennis*. More precisely, we showed that, depending on the total amount of sperm they received and stored in their spermatheca, *N. vitripennis* females produce various numbers of females relative to males along their lifetime. Although all types of females (not-limited, partially-limited and very-limited in sperm) produced a similar total number of offspring, the very limited ones had, as expected, fewer daughters. In addition, depending on their level of sperm limitation, females seem to run out of spermatozoa more or less prematurely, hence affecting their distribution of daughters' production along their oviposition events.

Male ejaculate, transferred to females upon mating, is composed of spermatozoa and seminal fluid: both are known to affect females' behaviors and physiology in insects (Avila et al., 2011, Gillott, 2003). In our study, we used two methods to obtain sperm-limited females: heat-stressed and previously mated males. In particular, females mated with 34°C heat-stressed males or multiply mated males received a similar amount of spermatozoa, corresponding to around 40% of the sperm stock transferred by control males. Nevertheless, stress or life history traits cannot only alter spermatozoa number but also seminal fluid composition and quantity. Seminal fluid proteins are involved in sperm storage parameters by impacting the viability of stored sperm or their release from female storage organs (Avila et al., 2011). A modification of seminal fluid composition after heat stress during male development can occur and may be responsible for the modification of female sex allocation. For instance, heat stress on rams affects their seminal plasma proteome, concomitantly with variations in semen parameters (Rocha et al., 2015). Furthermore, the quantity of seminal fluid transmitted to females can vary with the number of successive matings males previously had. In *Drosophila melanogaster*, five matings in rapid succession lead to the depletion of seminal fluids but not of spermatozoa (Linklater et al., 2007). Due to the potentially different underlying mechanisms, heat stress and multiple matings are likely to affect the composition and quantity of seminal fluid in different ways. In our study, we observed a similar pattern of sex allocation in both types of partially-

limited females (i.e. females mated with 34°C heat-stressed males and multiply mated males). Therefore, our results suggest that spermatozoa number in spermatheca is more likely to explain the sex allocation observed.

Here, we showed that all types of females produced a similar number of offspring during their lifetime. This indicates that sperm limitation did not affect their egg production, as observed in other sperm-depleted parasitic wasps, such as *Bracon hebetor* (Ode et al., 1997). Like other synovigenic insects, females of *N. vitripennis* emerge with only a fraction of their mature eggs, the others being matured along their adult life. Oviposition and adult nutrition, including host feeding, are well known to influence the rate of egg production in these synovigenic parasitoids (e.g. (Jervis & Kidd, 1986, Rivero-Lynch & Godfray, 1997)). In our experiments, females were provided with two hosts per day. Under these conditions, we observed that offspring production occurred in two main waves. We reported a first peak of production between the third and sixth oviposition days, in accordance to Edwards (Edwards, 1954). Offspring production then declines until a second smaller peak that starts after the eleventh oviposition day. This phenomenon most likely relates to the dynamic of egg maturation and oosorption of *N. vitripennis* under our experimental conditions.

As predicted by the LMC theory (Hamilton, 1967), *N. vitripennis* females naturally produce a sex ratio highly skewed towards females when exploiting hosts alone (Grillenberger et al., 2008). Here, females mated with not-limited control males maintained a sex ratio of 90% of daughters throughout their oviposition events: their sperm stock appears to be largely sufficient since these females received twice as many spermatozoa as they need to produce their daughters in this experiment, as observed in many other species (Bressac et al., 2008). In contrast, the proportion of females produced per oviposition event decreased over time with sperm limitation. Depending on the quantity of sperm females received from males, the alteration of sex ratio appears more or less prematurely. In females with a very limited sperm stock (mated with 36°C heat-stress males), deviation in sex-allocation is observed as early as the first few ovipositions while in partially limited females (mated with 34°C heat-stress males and multiply mated males), it only starts just before or during the second wave of egg

production. Females mated with 36°C heat-stressed males received a particularly low sperm stock: a third of those necessary to produce as many daughters as females mated with control males. As a result, they produced, in total, fewer daughters and more sons than females mated with control males. Interestingly, from the third oviposition day, although they had enough spermatozoa left and could maintain an optimal sex ratio (according to LMC) for a couple of additional ovipositions, they spread their daughters across 4 to 5 oviposition days. A similar trend is observed in partially-limited females during the second wave of egg production. Indeed, during this period, the increase in offspring number was mainly due to a higher production of sons in sperm-limited females (suppl. data), while it was due to a higher production of daughters in control females. This suggests that partially-limited females started running out of sperm quite late in our experiments. Most probably, these effects would have been observed earlier and with a stronger magnitude if females had been provided with more hosts per day.

The observed biased sex ratio can be the result of a physiological constraint related to sperm limitation. For example, in honeybees, queens with a low number of spermatozoa left in their spermatheca are forced to lay unfertilized eggs, which are considered as a honest signal for workers to replace them in the colony (Baer et al., 2016). In *N. vitripennis*, females are likely to use all the spermatozoa stored in their spermathecal to the very last, as observed in other Pteromalidae, *Dinarmus basalis* (Chevrier & Bressac, 2002). However, the dynamic of spermatozoa release from the spermatheca may depend on their remaining quantity. Indeed, in wasps, the spermathecal pump consists of a sphincter of concentric muscle fibers (Pascini & Martins, 2016), suggested to be involved in controlling the release of spermathecal content during fertilization (Baer et al., 2016). At the time of fertilization, muscle fibers of the sphincter relax allowing the release of sperm into the oviduct where fertilization occurs (Pascini & Martins, 2016). The quantity of sperm present in the spermatheca may thus affect the probability of spermatozoa release: the fewer spermatozoa there are, the less likely they may exit the spermatheca. As a result, the production of daughters would be constrained to slow down progressively over successive ovipositions, as females become sperm depleted.

Nevertheless, females may be able to control the opening of the sphincter as well as how long it remains open. Various molecules could be involved in this mechanism, such as the two neuromodulators, the biogenic amines tyramine and octopamine, shown in *Drosophila melanogaster* (Avila et al., 2011). In *N. vitripennis*, the glucose deshydrogenase has also been suggested as required for the successful release of sperm from the spermatheca (Pannebakker et al., 2013). Females can thus adjust their use of sperm, hence their sex allocation, according to the situation (Grillenberger et al., 2008). The observed deviance in sex ratio would therefore reflect females' strategic allocation of their limiting sperm stock. The fact that females, very limited in sperm, produced fewer daughters than control females over the first few ovipositions, despite having enough spermatozoa to do so, is reminiscent of a bet-hedging strategy (Hopper, 1999, Philippi & Seger, 1989). Indeed, when living in unpredictable environments (e.g. varying in environmental conditions, predation risks, probability of finding high quality mates, hosts or food), organisms can be expected to maximize their fitness by adopting behaviors that allow them to spread their risks in space and time (Moreau et al., 2017). This would be even more marked when organisms deal with the allocation of limiting resources. Here, the reduced use of sperm (hence, of daughters produced) over the first few ovipositions may be advantageous for females mated with 36°C heat-stressed males. Being highly sperm-limited, they would only be able to produce a high number of daughters on a very few hosts. Because hosts can be destroyed by predators or unfavorable environmental conditions, the risk that no progeny would develop is not negligible. Under such conditions, "not putting all their eggs in one basket" appears adaptive (diversified risk-spreading strategy; (Hopper, 1999, Philippi & Seger, 1989). Nevertheless, and despite spreading their daughters over several hosts, sperm-limited females have allocated more of them in the first few hosts. It suggests that females would also respond to the risk of not finding any additional suitable hosts in the future. It would thus favor females that "always play it safe" by investing more sperm on the first hosts, in case they do not find any other (conservative bet-hedging, (Hopper, 1999, Philippi & Seger, 1989). When sperm-limited, *N. vitripennis* females therefore seem to

adjust optimally their sperm use by presenting a combination of both strategies, evocative of the optimal risk-spreading strategy described by (Olofsson et al., 2009).

Finally, it would be interesting to discriminate whether the observed modification of sex allocation results from a physiological constraint (i.e the availability of sperm exiting the spermatheca constrains egg fertilization) or a behavioral strategy (i.e. females strategically allocate the sperm they receive) of these females. To do so, we could try to precisely measure the oviposition behavior: in certain parasitoid species, such as the two gregarious parasitoids *Anaphes victus* and *A. listronoti*, abdominal vibrations and the pause following them are slightly longer when eggs are fertilized (Van Baaren et al., 1995). If a similar link between certain oviposition behavioral items and offspring sex were to exist in *N. vitripennis*, we could identify when females 'try' to lay daughters and then verify the sex of these offspring. In case of a physiological constraint, longer oviposition behaviors would not directly translate into daughter production. Whether sperm-limited females show this behavior in situations where they would oviposit daughters if not sperm-limited remains to be seen.

In conclusion, external phenomena altering male fertility, such as intense heat-stress, could have consequences on the dynamic of daughters' production and potentially impact the parasitic efficiency of those beneficial insects.

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477 **Tables**

478 **Table 1:** Mean number (\pm standard deviation) of spermatozoa stored in *N. vitripennis* female
 479 spermatheca after mating with a control or a sperm-depleted male.

Females mated with	Sperm stored in spermatheca	Sample size	Reference	Group of females
Control males	750 \pm 155 a	N=13	(Chirault et al., 2015)	Not-limited
Multiply-mated males	253 \pm 134 b	N=13	(Chirault et al., 2016)	Partially-limited
34°C heat-stressed males	291 \pm 190 b	N=14	(this study)	Partially-limited
36°C heat-stressed males	144 \pm 105 c	N=13	(Chirault et al., 2015)	Very-limited
ANOVA test	$F_{3,49} = 41.4, P < 0.001$			

480 Different letters indicate a significant difference at $P < 0.001$.

481 **Table 2:** Total number of offspring and daughters produced (mean \pm SEM) by each type
 482 of females over the course of the experiment

Group of females	Females mated with	Total No. offspring	Total No. daughters
Not-limited	Control males	471 \pm 29	420 \pm 27 a
Partially-limited	Multiply-mated males	559 \pm 47	448 \pm 29 a
Partially-limited	34°C heat-stressed males	452 \pm 48	355 \pm 42 a
Very-limited	36°C heat-stressed males	416 \pm 35	107 \pm 17 b
<i>Kruskal-Wallis test</i>		H = 4.80, <i>P</i> = 0. 19	H = 36.96, <i>P</i> < 0.001

483 Different letters indicate a significant difference at *P* < 0.001.

Table 3: Statistical results from mixed-effect linear models investigating the effect of the type of males that inseminated females (i.e. control, multiply-mated, 34° and 36°C heat-stressed males) and the day of oviposition on the total number of offspring laid by females and the number of daughters produced. Statistical results from mixed-effect logistic models investigating the effect of the same parameters on the odd sex ratio produced by females. Female identity was used as a random-effect term.

Terms	df	No. offspring			No. daughters		Odd sex-ratio		
		χ^2	P-value		χ^2	P-value	df	χ^2	P-value
Type of males	3	3.60	0.31		143.31	<0.001	3	120.31	<0.001
Oviposition days	14	160.88	<0.001		183.31	<0.001	13	210.07	<0.001
Males x ovip. days	41	87.02	<0.001		140.06	<0.001	39	521.03	<0.001

489

Figure legends

Figure 1: Mean number (\pm SEM) of offspring (A) and daughters (B) and sex-ratio, in terms of the proportion of females (C) produced per oviposition day by females inseminated by either a control male or a sperm-limited male (i.e. multiply-mated, 34°C or 36°C heat-stressed males). NS: Non-significant, *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$. Statistical results noted above the graphs indicate whether the values per oviposition day were significantly different between the 4 types of females. Different letters indicate a significant difference at $\alpha=0.05$. Letters in brackets indicate that the three closest values were not significantly different. Because females inseminated by a multiply mated male did not lay offspring on the 15th oviposition day, we restricted the sex-ratio comparison to the first 14th oviposition days.