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

2

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

5

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10 **Abstract**

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

26

27 **Key-words:** Heat stress, multiple mating, sex ratio, sperm use, sperm stock, offspring production
28 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.

54 Blaul & Ruther, 2011, Chihrane & Laugé, 1994, Chirault et al., 2015, Lacoume et al., 2006, Lacoume et
55 al., 2007, Lacoume et al., 2009, Nguyen et al., 2012, Rohmer et al., 2004). These cumulative effects
56 lead males to transfer reduced ejaculates to females, although they continue to attract receptive
57 females for mating (Damiens & Boivin, 2005, Assem, 1986). In monandrous species, which represent
58 80% to 85% of hymenopteran parasitoid species (Gordh & DeBach, 1978, Ridley, 1993), the costs of
59 mating with such males is particularly high, because sperm-limited females are then forced to produce
60 suboptimal sex ratio by laying more sons than predicted by LMC even in good quality hosts (host quality
61 model predicts that males should mainly be laid in poor quality hosts (Charnov, 1982). This raises the
62 question of how females use this limiting resource over the course of their successive ovipositions.
63 Does sperm limitation totally constrain female sex allocation or can female strategically allocate this
64 resource and adjust the sex ratio of their offspring according to their level of sperm limitation? We
65 addressed these questions in *Nasonia vitripennis* (Hymenoptera: Pteromalidae), a gregarious
66 parasitoid attacking pupae of different Dipteran species (Whiting, 1967).

67 Sex allocation in *N. vitripennis* has been the subject of many studies (Godfray et al., 1994,
68 Shuker et al., 2007, Shuker & West, 2004, Werren, 1980). Females adjust their offspring sex ratio in
69 accordance to the predictions of the Local Mate Competition theory (Hamilton, 1967). Mated *N.*
70 *vitripennis* females produce strongly female-biased sex-ratio (~88% females) when exploiting a host
71 patch alone (Grillenberger et al., 2008). However, when they mate with non-virgin males, the number
72 of spermatozoa transferred decreases with the number of females a male has previously inseminated
73 (Chirault et al., 2016, Ruther et al., 2009). Females also receive a low number of spermatozoa after
74 mating with males that were exposed to a heat stress during juvenile development (Chirault et al.,
75 2015). Males exposed to high temperature indeed produce a reduced number of spermatozoa
76 throughout their life (Chirault et al., 2015, Chirault et al., 2016). When females are mated with males
77 exposed to 36°C for 24h during the pupal stage, they had about 80% fewer spermatozoa in their
78 spermatheca than those mated with control males (Chirault et al., 2015). Being monandrous (Burton-
79 Chellew et al., 2007), choosing a high quality male is key for *N. vitripennis* female reproductive success

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

86 In the context of climate change and the increasing risk of animals suffering heat-stress, we
87 investigated to what extent different levels of sperm limitation due to different heat-stresses (34°C &
88 36°C) affect females' offspring production and sex allocation. Is females' sex-ratio fully constrained by
89 sperm quantity, or can they adjust their sperm use decisions, hence sex allocation, host by host? To
90 test this, we compare the sex allocation of females presenting three levels of sperm limitation (not-
91 limited, partially-limited and very-limited) on each host they parasitized. To disentangle the potential
92 reduction of sperm quality after a heat stress exposure from that of sperm quantity, we also explored
93 the clutch size and sex-ratio produced by females that were partially sperm limited because they
94 mated with multiply mated males.

95

96 **Material and methods**

97 ***Insect rearing***

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

105 To obtain virgin individuals of both sexes, pupae were collected by opening the host puparium
106 1 to 4 d before emergence. Each wasp pupa was individually maintained in a 2 mL tube, closed with a
107 cotton ball. Emergences were checked every day and females were used in experiments the day of
108 their emergence. To generate additional males for experiments, virgin females were provided with
109 hosts. The day of their emergence, virgin males issued from such broods were randomly assigned to
110 one of the different treatments (see below for details).

111

112 ***Experimental design***

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

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

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

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

151 ***Statistical analyses***

152 To define the different categories of females, we compared the number of spermatozoa they
153 stored in their spermatheca according to the type of males they mated with (i.e. either a control male
154 (data coming from Chirault et al., 2015), a multiply-mated male (data coming from Chirault et al.,
155 2016), a 34°C heat-stressed male (data from this study) or a 36°C heat-stressed males (data coming
156 from Chirault et al., 2015)) using an ANOVA test followed by Tukey post-hoc comparisons.

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

168 We further tested the effect of sperm stored by females on their odd sex ratio (in terms of the
169 proportion of daughters produced) for each oviposition day using a mixed-effect logistic regression
170 (function *glmmPQL*) fitted to a quasibinomial error distribution. The type of males they mated with
171 and the day of oviposition were used as fixed-effect explanatory variables and female identity as a
172 random-effect term. When the interaction term between the *type of males* and *oviposition days* was
173 significant, we compared the odd sex ratio produced by the females mated with the different types of
174 males for each oviposition day performing ANOVAs followed by Tukey post-hoc comparisons. Because
175 females inseminated by a multiply mated male did not lay offspring on the 15th oviposition day, we
176 restricted our comparison to the first 14th oviposition days. Finally, we investigated whether female
177 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)

180 **Results**

181 *Sperm stored by females*

182 The quantity of sperm that females stored in their spermatheca varied with the type of males they had
183 mated with (Table 1). Sperm stock of females mated with a 34°C heat-stressed and a multiply-mated
184 male did not differ significantly and was intermediate compared to the two other types of females
185 (Table1). We therefore classed them into 3 groups: not-limited, partially-limited and very-limited in
186 spermatozoa. The comparison of offspring production and sex allocation of both types of partially-
187 limited females allowed controlling for a potential effect of heat stress on the quality of the sperm
188 transferred by males.

189 *Offspring production and sex allocation*

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

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

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

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

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

211 *Female survival*

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

214 Discussion

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

223 Male ejaculate, transferred to females upon mating, is composed of spermatozoa and seminal
224 fluid: both are known to affect females' behaviors and physiology in insects (Avila et al., 2011, Gillott,
225 2003). In our study, we used two methods to obtain sperm-limited females: heat-stressed and
226 previously mated males. In particular, females mated with 34°C heat-stressed males or multiply mated
227 males received a similar amount of spermatozoa, corresponding to around 40% of the sperm stock
228 transferred by control males. Nevertheless, stress or life history traits cannot only alter spermatozoa
229 number but also seminal fluid composition and quantity. Seminal fluid proteins are involved in sperm
230 storage parameters by impacting the viability of stored sperm or their release from female storage
231 organs (Avila et al., 2011). A modification of seminal fluid composition after heat stress during male
232 development can occur and may be responsible for the modification of female sex allocation. For
233 instance, heat stress on rams affects their seminal plasma proteome, concomitantly with variations in
234 semen parameters (Rocha et al., 2015). Furthermore, the quantity of seminal fluid transmitted to
235 females can vary with the number of successive matings males previously had. In *Drosophila*
236 *melanogaster*, five matings in rapid succession lead to the depletion of seminal fluids but not of
237 spermatozoa (Linklater et al., 2007). Due to the potentially different underlying mechanisms, heat
238 stress and multiple matings are likely to affect the composition and quantity of seminal fluid in
239 different ways. In our study, we observed a similar pattern of sex allocation in both types of partially-

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

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

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

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

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

292 Nevertheless, females may be able to control the opening of the sphincter as well as how long
293 it remains open. Various molecules could be involved in this mechanism, such as the two
294 neuromodulators, the biogenic amines tyramine and octopamine, shown in *Drosophila melanogaster*
295 (Avila et al., 2011). In *N. vitripennis*, the glucose deshydrogenase has also been suggested as required
296 for the successful release of sperm from the spermatheca (Pannebakker et al., 2013). Females can thus
297 adjust their use of sperm, hence their sex allocation, according to the situation (Grillenberger et al.,
298 2008). The observed deviance in sex ratio would therefore reflect females' strategic allocation of their
299 limiting sperm stock. The fact that females, very limited in sperm, produced fewer daughters than
300 control females over the first few ovipositions, despite having enough spermatozoa to do so, is
301 reminiscent of a bet-hedging strategy (Hopper, 1999, Philippi & Seger, 1989). Indeed, when living in
302 unpredictable environments (e.g. varying in environmental conditions, predation risks, probability of
303 finding high quality mates, hosts or food), organisms can be expected to maximize their fitness by
304 adopting behaviors that allow them to spread their risks in space and time (Moreau et al., 2017). This
305 would be even more marked when organisms deal with the allocation of limiting resources. Here, the
306 reduced use of sperm (hence, of daughters produced) over the first few ovipositions may be
307 advantageous for females mated with 36°C heat-stressed males. Being highly sperm-limited, they
308 would only be able to produce a high number of daughters on a very few hosts. Because hosts can be
309 destroyed by predators or unfavorable environmental conditions, the risk that no progeny would
310 develop is not negligible. Under such conditions, "not putting all their eggs in one basket" appears
311 adaptive (diversified risk-spreading strategy; (Hopper, 1999, Philippi & Seger, 1989). Nevertheless, and
312 despite spreading their daughters over several hosts, sperm-limited females have allocated more of
313 them in the first few hosts. It suggests that females would also respond to the risk of not finding any
314 additional suitable hosts in the future. It would thus favor females that "always play it safe" by
315 investing more sperm on the first hosts, in case they do not find any other (conservative bet-hedging,
316 (Hopper, 1999, Philippi & Seger, 1989). When sperm-limited, *N. vitripennis* females therefore seem to

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

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

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

333

334

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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.

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

Terms	No. offspring			No. daughters		Odd sex-ratio		
	<i>df</i>	χ^2	<i>P</i> -value	χ^2	<i>P</i> -value	<i>df</i>	χ^2	<i>P</i> -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

490 **Figure legends**

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