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## **Sperm-depleted males influence the reproductive behavior of conspecifics**

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2 **Abstract**

3 In many insect species, sperm-depleted males (SDMs, i.e. males that have exhausted their  
4 sperm after a given number of matings) remain sexually active, and continue to mate females.  
5 Here we investigated the behavioral modifications that occur in both sexes of the parasitoid  
6 *Asobara tabida* Nees (Hymenoptera: Braconidae), after matings by fertile males and sperm-  
7 depleted males. We show that (i) virgin females, mated females, and females mated to a  
8 SDM exhibited different behaviors, and that (ii) males responded differently to females  
9 depending on whether the females had previously mated with an SDM or not. Our findings  
10 demonstrate that SDM influenced the reproductive behavior of both males and females,  
11 especially with regard to male responsiveness and female attractiveness. These findings are  
12 discussed in the context of adaptive behavior and fitness maximization in both males and  
13 females.

14 **Keywords**

15 Sperm-depleted male, hymenopteran parasitoids, mate choice, *Asobara tabida*, conspecific  
16 behavior

17

## 18 **Introduction**

19 Sperm is not an infinite and free-cost resource to produce. In polygynous species, mating  
20 several females successively in a short period of time may result in sperm depletion: The  
21 sperm supply can be drained after a given number of females mated (Wedell et al. 2002).  
22 Sperm depletion can be temporary (in synspermatogenic species) or definitive (in  
23 prospermatogenic species), depending on the ability of males to produce new sperm during  
24 their adult life (Damiens and Boivin 2006; Boivin 2013). Sperm-depleted males (SDMs) have  
25 been found in many taxa, such as mammals (Preston et al. 2001), birds (Pizzari et al. 2003),  
26 lizards (Olsson et al. 2004), crustaceae (Rubolini et al. 2007) and fish (Smith et al. 2009). In  
27 insects, sperm depletion occurs in many species and SDMs individuals remain sexually active  
28 as they are still able to attract receptive females and to mate them (Simmonds 1953; Laing  
29 and Caltagirone 1969; Gordh and DeBach 1976; Assem et al. 1979; Nadel and Luck 1985;  
30 Ramadan et al. 1991; Ode et al. 1995; Damiens and Boivin 2005; Rönn et al. 2008; Steiner et  
31 al. 2008). It was suggested that continuing to mate is an adaptive strategy of SDMs to  
32 decrease the fitness of fertile males (Damiens and Boivin 2006). The authors indeed  
33 demonstrated that *Trichogramma evanescens* Westwood (Hymenoptera: Trichogrammatidae)  
34 females have a decreased ability to store sperm after being mated by SDMs (Damiens and  
35 Boivin 2006). To continue to mate is thus viewed as a SDMs' strategy to increase their  
36 relative fitness comparing to this of other males. On the other hand, such behavior may persist  
37 over generations because of the lack of selective pressure counter-selecting it (SDMs can  
38 obviously not produce progeny anymore). Whatever the adaptive significance of the SDM  
39 mating behavior, its effect on the behaviors of conspecifics (both males and females) remains  
40 largely unknown.

41 Females' behaviors may be modified after copulation; after mating, females usually  
42 become refractory to subsequent copulations for a given time, even without sperm transfer

43 (Gillott 2003). This refractoriness to mating is caused by chemicals contained in the seminal  
44 fluids transferred during mating or by the male's behavior (Alcock 1994; Gillott 2003; Wedell  
45 2005; Yamane 2013). The transfer of chemicals to the mated female is therefore viewed as a  
46 male strategy to suppress the subsequent reproductive activity of females, thus decreasing the  
47 likelihood of sperm competition (Ejima et al. 2007). For example, Radhakrishnan et al. (2009)  
48 showed that the transfer of seminal fluid in *Bactrocera tryoni* Froggatt (Diptera: Tephritidae)  
49 during mating induces female sexual inhibition. In this case, the behavior of the mated  
50 females is modified by SDM resulting in fewer opportunities for other males to access them.  
51 In many species, females mated to a SDM become less receptive to mating than virgin  
52 females, suggesting that these females no longer invest in pheromone biosynthesis after  
53 mating (Steiner et al. 2008). We however note that in at least another species, females mated  
54 to a SDM remain sexually active (Damiens and Boivin 2006).

55         Male mating may also modify the behavior of conspecific males. Males are known to  
56 be choosy just like females (Wedell et al. 2002), and the females' mating status influences  
57 male choice. For example, the male preferences for virgin females and/or for females that  
58 they have never previously encountered have been observed in many insects species  
59 (Bonduriansky 2001; Wedell et al. 2002; Ferveur 2005; Bateman and Fleming 2006;  
60 Everaerts et al. 2010). Sexual pheromones transferred by males during mating can mark  
61 females as mated, making them less attractive to other males (Findlay et al. 2008). In  
62 *Drosophila*, mating induces changes in the female cuticular profile, which reduces the time  
63 spent in copulation by subsequent males (Friberg 2006; Everaerts et al. 2010). Previous  
64 mating can therefore influence the choice of other males, which generally prefer to mate with  
65 virgin females.

66         Because all the behavioral modifications detailed above do not require sperm transfer,  
67 such modifications are likely to occur after mating by a SDM. The aim of our study was thus

68 to investigate the behavioral consequences of SDMs mating behavior in both sexes. In this  
69 system, conflict between the sexes arises, as females mated to a SDM are still unfertilized and  
70 so would gain by remaining attractive to other males in order to ensure egg fertilization. On  
71 the other hand, SDMs would gain by decreasing the attractiveness of the females they mated  
72 in order to reduce other males' opportunity to access them. Given that, we hypothesize that (i)  
73 females mated to a SDMs should be as receptive as virgin females in order to get access to  
74 further insemination, (ii) males should be more attracted to virgin females than females mated  
75 to a SDM and mated females.

76 To test these hypotheses, we used the parasitoid *Asobara tabida* Nees (Hymenoptera:  
77 Braconidae), a haplodiploid species in which sons result from unfertilized eggs while  
78 daughters result from fertilized eggs, allowing us to determine if the females had been  
79 successfully inseminated from the resulting sex ratio obtained – the presence of at least one  
80 daughter in the progeny is a proof of insemination. *Asobara tabida* males exhibit a  
81 stereotyped courtship behavior consisting of wing fanning, antennation, head movements, and  
82 leg tapping (Matthews 1975; Carton et al. 1986; Dufour et al. 2012). No postcopulatory  
83 behavior is reported for this species (Stökl et al. 2014). *Asobara tabida* males are generally  
84 sperm-depleted after a large number of matings (about 13 matings), but continue to mate after  
85 sperm depletion (Louâpre, Dufour, personal observation). Chemical communication is of  
86 great importance for mating as *A. tabida* males respond to chemicals deposited by females  
87 and increase their residence time in a patch previously exposed to females (Dufour et al.  
88 2012). More recently, Stökl et al. (2014) highlighted the remarkable diversity of the sex  
89 pheromones of males and females in *A. tabida*. They showed that mating behavior in this  
90 species is regulated by a complex combination of chemicals rather than by a single molecule.

91 **Materials and Methods**

92 *Insect rearing and general methods*

93 The *Asobara tabida* strain used in this study was collected in Chasse-Sur-Rhône (France,  
94 45°34'N, 4°48'E) in September 2008, and reared in the laboratory on second-instar  
95 *Drosophila subobscura* hosts in a controlled-climate room (20°C, 60% relative humidity, and  
96 16:8 h Light:Dark). A few days before emergence, parasitized pupae from the culture were  
97 isolated in gelatin capsules (0.68 mL) to obtain virgin parasitoids of known age. Freshly  
98 emerged males and females were kept separately in plastic tubes (h = 10 cm, Ø = 2.5 mm) and  
99 fed with diluted honey solution.

100 *Choice tests*

101 Experiments were performed in the controlled-climate room used for the rearing. Males were  
102 1-2 days old and females were an equal mixt of 1-4 days old (the two females used in each if  
103 the choice test were of comparable ages in order to avoid potential confounding effect of age).  
104 A light table was used to maintain the individuals on the bottom of a Petri dish (Ø = 90 mm)  
105 by phototaxis. All experiments were video-taped for a period of 15 minutes, and analyzed  
106 using the computer software package The Observer v3.0 (Noldus, Wageningen, The  
107 Netherlands). During each choice test, we noted (i) the male behavior (courtship, contact with  
108 the female, mating) (ii) the identity of the female toward which the male directed its behavior,  
109 and (iii) the behavior of the female toward the male (i.e. approaches and escapes, acceptance  
110 or rejection). The identity of the female toward which the first courtship was directed was also  
111 noted as a proxy of the male preference before any reaction from the female. Mating is indeed  
112 the result of both active and apparent choices of the two sexes and the first courtship give  
113 supplementary information compared to the mating with a given female (Martel and Boivin  
114 2011).

115 *Experiment 1: Female mated to a SDM vs. virgin female*

116 In this experiment, a virgin male was placed in the presence of both a female mated to a SDM  
117 and a virgin female in order to find out whether the male would perceive them as different  
118 resources. To obtain a female mated to a SDM, a single male mated a sequence of 30 virgin  
119 females over a period of 6 hours (from rank-1 to rank-30 females). Although *Asobara tabida*  
120 males become sperm-depleted after about 13 matings (Louâpre, Dufour, personal  
121 communication), we used females from rank-24 to rank-29 and we isolated the rank-23 and  
122 rank-30 females in a plastic tube containing a substrate of agar-nipagine and 20 second-instar  
123 *D. subobscura* larvae. The sex-ratio produced was noted, and only data from true SDMs and  
124 females mated to an actual SDM – whose offspring had a sex-ratio of 1 (i.e. male-only  
125 progeny) – were used for the analysis. In the rearing conditions, inseminated females indeed  
126 lay at least several females in a dozen of hosts, so that a male-only progeny obtained in 20  
127 hosts is likely to be a strong clue for an absence of fertilization of the females. One female  
128 mated to a SDM (only females mated to different SDMs were used for each of the repetitions)  
129 and virgin female were then placed in the same clean Petri dish. A virgin male was introduced  
130 at the beginning of the experiment and placed equidistant from the two females. Thirteen dual  
131 choice tests were performed.

132 *Experiment 2: Female mated to the same SDM vs. virgin female*

133 In this experiment, each SDM was placed in the presence of a female he had previously mated  
134 without sperm transfer (randomly selected from rank-24 to rank 29 in the sequence of  
135 copulations) and a virgin female. Both the female mated to the SDM and the virgin female  
136 were placed in the same clean Petri dish. The SDM was introduced at the beginning of the  
137 experiment and placed equidistant from the two females. Eleven dual choice tests were  
138 performed.



139 *Experiment 3: Female mated to the same fertile male vs. female mated to another fertile male*

140 In this experiment, a fertile male was placed in the presence of a female he had previously  
141 mated and a female that had been mated by another male to determine whether males  
142 recognize a female they have previously mated. Ten minutes before the choice test, two virgin  
143 males each mated a virgin female in separate plastic tubes (h = 10 cm,  $\varnothing$  = 2.5 mm). The two  
144 mated females were then placed in the same clean Petri dish. One of the two males used was  
145 introduced at the beginning of the experiment and placed equidistant from the two females.  
146 Twenty-two dual choice tests were performed.

147 *Statistical analyses*

148 Statistical analyses were performed using R 2.15.0 software at the critical level  $\alpha = 0.05$ . The  
149 male choices were compared to a 50:50 probability (i.e. no preferential choice) using an exact  
150 binomial test. The mean numbers of the types of behavior exhibited by the males and females  
151 were analyzed using Wilcoxon tests. Because both virgin females and females mated to a  
152 SDMs were used in the first two experiments, the occurrence of approaches and escapes  
153 during these two experiments were analyzed by GLM-Poisson procedure, using the mating  
154 status of the females (i.e. virgin, pseudo-virgin, or mated) and of the males (fertile or sperm-  
155 depleted) as factors. Multiple comparisons were then performed using the glht function of R  
156 (multcomp package).

157 **Results**

158 *Experiment 1: female mated to a SDM vs. virgin female (fig. 1)*

159 The two types of females exhibited the same number of approaches and escapes toward the  
160 virgin male (respectively  $W = 82$ ,  $P = 0.915$  and  $W = 78$ ,  $P = 0.753$ ) (fig. 1.b). Virgin males  
161 did not direct their first courtship or their first mating toward one female type rather than the

162 other (Binomial test, respectively  $P = 0.581$  and  $P = 0.179$ ) (fig. 1.a). During the course of the  
163 experiment, the males exhibited on average the same number of courtships and contacts with  
164 females mated to a SDM and virgin females (respectively,  $W = 68$ ,  $P = 0.401$  and  $W = 64$ ,  $P =$   
165  $0.288$ ) (fig. 1.c). They tended to mate the originally virgin females more frequently than the  
166 SDM-mated females during the course of the experiment, but this difference was not  
167 significant probably due to the weak number of repetitions ( $W = 52.5$ ,  $P = 0.055$ ) (fig. 1.c).

168 *Experiment 2: Female mated to the same SDM vs. virgin female (fig. 2)*

169 Virgin females exhibited more approaches toward the SDM than the females previously  
170 mated by him ( $W = 26.5$ ,  $P = 0.026$ ), while the number of escapes from the SDM did not  
171 differ between both groups of females ( $W = 46$ ,  $P = 0.352$ ) (fig. 2.b). The first courtship was  
172 not significantly exhibited by SDMs toward one type of females over the other (Binomial test,  
173  $P = 0.227$ ) (fig. 2.a). On average SDMs directed a similar number of courtships toward  
174 females mated by them and virgin females ( $W = 35$ ,  $P = 0.401$ ) (fig. 2.c). However, they  
175 exhibited more contacts with virgin females than with females they previously mated ( $W =$   
176  $29$ ,  $P = 0.037$ ). They mated the virgin females before the females they previously mated  
177 (Binomial test,  $P = 0.012$ ; fig. 2.a), and mated the originally virgin females more frequently  
178 than the SDM-mated females during the course of the experiment, even though she becomes  
179 mated after the first mating ( $W = 13.5$ ,  $P < 0.001$ ; fig. 2.c).

180 *Experiment 3: Female previously mated by the same fertile male vs. female previously mated*  
181 *by another fertile male (fig. 3)*

182 The two types of females exhibited the same number of approaches and escapes toward the  
183 fertile male ( $W = 299$ ,  $P = 0.104$  and  $W = 199$ ,  $P = 0.313$ , respectively) (fig. 3.b). Although  
184 fertile males directed their first courtship toward females previously mated by another male  
185 more often than toward the ones they had mated themselves (Binomial test,  $P = 0.004$ ) (fig.

186 3.a), they exhibited overall the same number of courtships and contacts toward both females  
187 ( $W = 184.5$ ,  $P = 0.171$  and  $W = 211$ ,  $P = 0.458$ , respectively) (fig. 3.c). No mating was  
188 observed during this experiment.

### 189 *Female behavior across the experiments (fig. 1.b and 2.b)*

190 The behavior of virgin females and females mated to a SDM was compared between  
191 experiments 1 and 2. The number of approaches by females differed depending on their  
192 mating status (GLM-Poisson,  $F_{1,80} = 4.007$ ,  $P = 0.045$ ), but not on the male mating status  
193 (GLM-Poisson,  $F_{1,79} = 0.442$ ,  $P = 0.51$ ): females mated to a SDM exhibited more approaches  
194 than virgin females, regardless of the mating status of the males ( $z = -1.997$ ,  $P = 0.046$ ). The  
195 number of escapes differed between females, but differently depending on the mating status  
196 of the male (GLM-Poisson, female mating status: male mating status,  $F_{3,78} = 2.992$ ,  $P =$   
197  $0.029$ ): females mated to a SDM exhibited the same number of escapes from virgin males and  
198 SDMs ( $z = -0.205$ ,  $P = 0.997$ ), while virgin females exhibited more escapes from SDMs than  
199 virgin males ( $z = 2.624$ ,  $P = 0.043$ ).

## 200 **Discussion**

201 We showed that (i) virgin females, mated females, and females mated to a SDM exhibited  
202 different behaviors, and that (ii) the mating status of the females influences the behaviors of  
203 males. Different behaviors were observed according to the reproductive status of the two  
204 sexes, although the majority of the females were of the same mating status at the end of the  
205 experiment. As pointed out by Martel and Boivin (2011), mating depends not only on the  
206 choice of the individual (true choice), but also on the response of the partner (apparent  
207 choice). Accordingly, we showed that the mating pattern in this species can be influenced by  
208 the SDM's behavior, which in turn impacts both males' and females' choice. Our results thus  
209 demonstrate that SDMs influenced the male responsiveness and female attractiveness.

210 Sperm-depleted males influence the subsequent behavior of both the females they  
211 have mated and virgin females. Females mated to a SDM were more attracted to males than  
212 virgin females whatever the reproductive status of the males. Moreover, virgin females  
213 exhibited more escapes from SDMs than from virgin males. Taken together, these findings  
214 show that (i) females mated to a SDM were more reproductively active than virgin females,  
215 and that (ii) SDMs are not as attractive for females as virgin males. Females mated to a SDM  
216 should be as least as active as virgin females in order to compensate for the unsuccessful  
217 insemination (Arnqvist and Nilsson 2000; Hosken et al. 2001; Chapman et al. 2003; Tregenza  
218 et al. 2006). Our results are consistent with this and demonstrate that even if SMDs  
219 transferred seminal fluid to the females, this did not inhibit female sexual receptivity as it was  
220 demonstrated for other species (Leopold 1976; Avila et al. 2011). Female *A. tabida* mated to a  
221 SDM are thus receptive to mating, as has been shown for females *Trichogramma evanescens*  
222 mated to a SDM (Damiens and Boivin 2006). Moreover, females mated to a SDM seemed to  
223 exhibit adaptive behavior, because they reduced the number of approaches they made toward  
224 SDMs; whatever the mechanism behind this post-mating effect, females mated to a SDM  
225 behave as if they can “learn” and consequently modify their behavior. This is comparable to  
226 other types of behavior exhibited by females in order to ensure that their eggs are fertilized  
227 (reviewed by Rhainds 2010).

228 The behavior of males is also influenced by the reproductive status of the females;  
229 virgin females, females mated by a SMD and mated females do not elicit the same behavioral  
230 response from males according to the mating status of the males. Our results did not show any  
231 significant effect of the reproductive status of the females on the behavior of virgin males  
232 (experiment 1). We however noted a tendency of virgin males to exhibit their first courtship  
233 and mating toward virgin females, and the small sample size could have led to non-significant  
234 results. SDMs, however, did exhibit their first courtship and mating toward virgin females

235 over females they previously mated. They also exhibited more contacts toward virgin females  
236 over females they mated (experiment 2). These results demonstrate that females mated to a  
237 SDM are not perceived as virgin females by the males. The preferential choice of SDMs for  
238 virgin females rather than females mated to a SDM – even though females mated to a SDM  
239 continue to be receptive to mating – suggests a decrease in the attractiveness of females mated  
240 to a SDM.

241 Many cues can be used by males to discriminate between virgin females and females  
242 mated to a SDM, and chemicals must play a major role (Thomas 2011). In bumblebees and  
243 parasitic wasps, sexual pheromones are indeed known to carry information about mating  
244 status (Lloyd 1981; Ode et al. 1995). In *Drosophila*, the cuticular profiles of females change  
245 after mating (Steiger et al. 2008). Moreover, the mating behavior of *Asobara tabida* – like  
246 many other insect species – is based on a complex and dynamic infochemical emission, and  
247 on the perceptions of both males and females (Stökl et al. 2014). *Asobara tabida* males may  
248 thus perceive and respond to chemical changes in the mated females (with or without sperm  
249 being stored), which enable them to discern the female’s reproductive status.

250 Males could be more attracted to virgin females than to females mated to a SDM  
251 because they never encountered the virgin females they faced with (the so-called ‘Coolidge  
252 effect’) (Wilson et al. 1963; Dewsbury 1981). Based on individual marking recognition,  
253 Steiner et al. (2008) have shown that males of the burying beetle *Nicrophorus vespilloides*  
254 Herbst (Coleoptera: Silphidae) use female cuticular patterns to discriminate between novel  
255 females and those they have previously inseminated. In our study, such a “Coolidge effect”  
256 may be responsible for the choice of males faced by two mated females (experiment 3), and  
257 the choice of a SDM facing virgin females and females mated to a SDM (experiment 1), but  
258 not for that of virgin males in the first experiment. Indeed, the virgin males used in our study  
259 had never encountered any females. The change in the behavior of the males and females may

260 therefore involve different mechanisms, and do not depend on sperm transfer.

261 To conclude, our study showed that the mating process in *A. tabida* depends on the  
262 behavior of both sexes, and is not a simple and unidirectional choice of one sex toward the  
263 other. *Asobara tabida* males thus actively choose their mates on the basis of mating status, but  
264 females can accept or reject the interested males. SDMs and females mated to a SDM behave  
265 in different ways towards fertile males, and virgin or mated females, respectively. As other  
266 authors have suggested, the modification of male and female behavior as a result of mating  
267 could be a way of improving the fitness of SDMs (Damiens and Boivin 2006) by decreasing  
268 the likelihood of subsequent mating by other males through sperm competition avoidance.  
269 Many hypotheses have been advanced to explain the female mating failure in nature,  
270 including a lack of opportunity for mating (Rhainds 2010). The mating behavior of SDMs  
271 may contribute to this phenomenon by making females mated to a SDM look like successfully  
272 mated females to other males.

273

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398 **Figure legends**

399 **Figure 1: Behaviors of virgin males (a, b), virgin females and females mated to a SDM**

400 **(c) during the first experiment.** (a) Proportion ( $\pm$  95 % confidence interval) of virgin males  
401 exhibiting their first courtship toward (top,  $n = 13$ ) and/or mated first with (bottom,  $n = 9$ ) the  
402 proposed females. (b) Mean number ( $\pm$  SE) of behavior exhibited by virgin males toward  
403 different types of females. (c) Mean number ( $\pm$  SE) of behavior exhibited by females toward  
404 virgin males. (ns.: not significant).

405 **Figure 2: Behaviors of SDMs (a, b), virgin females and females mated to the same SDM**

406 **(c) during the second experiment.** (a) Proportion ( $\pm$  95 % confidence interval) of SDMs  
407 exhibiting their first courtship toward (top,  $n = 11$ ) and/or mated first with (bottom,  $n = 11$ )  
408 the proposed females. (b) Mean number ( $\pm$  SE) of behavior exhibited by SDMs toward  
409 different types of females. (c) Mean number ( $\pm$  SE) of behavior exhibited by females toward  
410 the SDM. (ns.: not significant; \*  $P < 0.05$ ).

411 **Figure 3: Behaviors of fertile males (a, b), females mated to the same male or to another**

412 **male (c) during the second experiment.** (a) Proportion ( $\pm$  95 % confidence interval) of  
413 fertile males exhibiting their first courtship toward ( $n = 22$ ) the proposed females. (b) Mean  
414 number ( $\pm$  SE) of behavior exhibited by the SDM toward different types of females. (c) Mean  
415 number ( $\pm$  SE) of behavior exhibited by females toward the SDM. (ns. not significant; \*\*  $P <$   
416  $0.01$ ).

417

418 **Figures**

419 Figure 1

420 **Les paramètres nécessaires sont manquants ou erronés.**

421

422 Figure 2

423 **Les paramètres nécessaires sont manquants ou erronés.**

424

425 Figure 3

426 **Les paramètres nécessaires sont manquants ou erronés.**