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## **Reproductive behaviours in male parasitoids: from mating system to pairing pattern**

Inter and intra-individual variations in sexual behaviours and the impact of social environment on these behaviours in a male parasitoid wasp, *Venturia canescens*.

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## **Abstract**

It is now acknowledged that mating system is a dynamic characteristic within species or populations. Indeed, behavioural characteristics shaping the mating system can vary at the inter-individual level, providing raw material for sexual and natural selections, and at the intra-individual level, because of phenotypic plasticity and ontogeny of individual behaviours. Social environment is an additional source of variation in sexual behaviours. By focusing on males of the parasitoid wasp *Venturia canescens*, we assessed how sexual behaviours (courtship and mating) vary between and within individuals, considering males' size as inter-individual source of variation and age and mating experience as intra-individual sources of variation. Consequences of the variations in sexual behaviours at the individual level are appraised by quantifying the costs in longevity and offspring production incurred by (repeatedly) mated males. At the population level, we tested "agility hypothesis" predicting that smaller males win the male-male competition to access females, resulting in a non-random assortative mating by size and a negative sexual selection on body size. We showed that *V. canescens* males are polygynous and that mating incurred longevity cost. Old males had a higher probability to court females, but no mating advantage. Smaller males tended to have a higher mating probability, but size was not under sexual selection and there was a random mating pattern according to individual size. Male-male competition intensity did not influence pairing pattern but tended to influence mating dynamic. Overall, our results suggest that sexual behaviours tended to vary at the inter- and intra-individual levels and the effect of social environment remains to be more precisely described. These variations may impact individuals' reproductive success with potential ecological and evolutionary consequences that are discussed.

**Key-words:** male size, competition, agility hypothesis, assortative mating, sexual selection opportunity, *Venturia canescens*

## 1 **Introduction**

2 Mating system defines the number of sexual mates individuals of both sexes have during  
3 a breeding season (Shuker & Simmons 2014). The mating system of a species or a population  
4 results from individuals' ability to access mates and reproduce, and thus from individuals'  
5 sexual behaviours (Emlen & Oring 1977). The mating system, often considered as a fixed  
6 characteristic of a species or a population, is dynamic because individuals' sexual behaviours  
7 and their ability to access mates vary in space and time according different sources of variation  
8 (Shuster & Wade 2003). First, inter-individual variation in sexual behaviours and  
9 characteristics represents a raw material on which natural and sexual selections operate. Second,  
10 there is an intra-individual variation in sexual behaviour and characteristics linked to both  
11 phenotypic plasticity and ontogeny. Finally, the environment (both social and physical) that  
12 directly affects individuals' sexual behaviour and varies in space and time. These three sources  
13 of variation (inter-individuals, intra-individuals, and environmental) shape the evolution of  
14 mating systems (Thornhill & Alcock 1983). These sources of variation are well illustrated in  
15 insects (Godfray, 1994; Shuker & Simmons, 2014) showing for instance their impact on  
16 variability of sexual behaviours in males and in their ability to secure reproduction.

17 Inter-individual variations in reproductive opportunities are clearly linked to mate  
18 choice and intra-sexual competition for access to a partner. Under the classical sexual selection  
19 theory, males compete with each other for females, and females use signals from males to  
20 choose their partner (Thornhill, 1976; Metzger, Bernstein, et al., 2010a). Variation among males  
21 in their access to females thus depends on their competitiveness and attractiveness. Both may  
22 be determined by their body size or the size of their weapons (Beukeboom, 2018), their ability  
23 to find females (Metzger, Fischbein, et al., 2010b), their coloration (Hawkes et al., 2019), the  
24 vigour and duration of their parade (Balaban-Feld & Valone, 2017).

25 Intra-individual variations in reproductive opportunities are related to the change in  
26 individual's ability to access a partner during its reproductive lifetime. This change is primarily  
27 affected by ageing meaning that male's body condition and vigour decrease with age (Rockstein  
28 & Miquel, 1973). Ageing can also generate variation in production of male pheromones that  
29 are used as signals by females to choose their partner (Ingleby, 2015). In the butterfly *Bicyclus*  
30 *anyana* male sex pheromone composition varies with age, and females prefer mid-aged over  
31 young and old males (Nieberding et al., 2012). Performance of males, and thus their investment  
32 into ejaculates, are often predicted to decrease with age (senescence). Ejaculate quality  
33 diminishes with age in the tropical butterfly *Bicyclus anyana* (Kehl et al., 2015). Intra-  
34 individual variations in reproductive opportunities (e.g. number of courtships and matings) are

35 also associated to changes in reproductive cost (e.g. in terms of energy expenditure) during  
36 reproductive lifetime (likely not independent of age and experience). Numerous examples  
37 support the hypothesis that increasing the number of reproductive opportunities affects residual  
38 reproductive success of males. In the ladybird, *Menochilus sexmacutatus*, frequent mating  
39 attempts made by males result in reduction in their body weight, and males engaging in  
40 consecutive matings suffer a longer latency before a new mating (Shandilya et al., 2021).

41 Environmental variations, through social environment that corresponds to change in  
42 operational sex ratio (OSR), may influence the intensity in male-male competition (Emlen &  
43 Oring, 1977). These variations in social environment also interact with individual variations  
44 (both inter- and intra-individual) to indirectly affect males' ability to access a partner (Kelly,  
45 2018). In the bushcricket, *Poecilimon ampliatus*, male body condition and male social  
46 environment affect male acoustic signals used to attract females. Heavy males modify the  
47 characteristics of their songs in response to different social conditions, when competing against  
48 light rivals. At the opposite, light males show less plasticity in their acoustic signals in response  
49 to social conditions when they compete with heavy males (Anichini et al., 2018).

50 At the population level, variations in individual sexual behaviours frequently result, for  
51 instance through mate choice, in non-random pairing (Shuster & Wade, 2003) such as  
52 assortative mating patterns (Arnqvist et al., 1996). These latter may be based on several  
53 characteristics : individual colour (Mason, 1972), environmental rearing conditions  
54 (Geiselhardt et al., 2012) for an example in the mustard leaf beetle), or size which remains the  
55 most documented (Otronen, 1993, for an example in the yellow dung fly). Moreover, assortative  
56 mating patterns may be affected by the environmental conditions encountered. Indeed, social  
57 environment, by conditioning intensity of intra-sexual competition, sexual behaviours, and  
58 availability of the sexual partners, may thus influence the assortative mating patterns. In the ant  
59 *Tranopelta gilva* assortative mating occurs only when mating competition is weak (low ant  
60 density and a slightly male-biased sex ratio) (Moura & Gonzaga, 2018).

61

62 In this study, we assessed how sexual behaviours (mating and courtship characteristics)  
63 vary between and within males to better understand mating systems in the parasitoid wasp,  
64 *Venturia canescens* (Hymenoptera: ichneumonidae). Our first aim was to describe courtship  
65 and mating characteristics (latency, number and duration) in males. We focused on pre-  
66 copulatory behaviours because *V. canescens* females are monandrous (Collet et al., 2020;  
67 Metzger et al., 2010b), so that no sperm competition nor cryptic female choice occur. We  
68 expected that *V. canescens* males are polygynous since guarding behaviours have never been

69 reported in these wasps. More precisely, we predicted a scramble competition polygyny  
70 (according to Thornhill & Alcock, 1983 classification) since emerging females are widely  
71 dispersed (van Santen & Schneider, 2002), making it unlikely that males monopolise  
72 oviposition sites, i.e. “rendez-vous” sites where sexual partners encounter (Metzger, et al.,  
73 2010b). Moreover, polygynous systems should lead to differential costs in terms of post-mating  
74 residual longevity and offspring sired; the more males have access to reproduction, the shorter  
75 they live and the more daughter production may be impaired. As an inter-individual source of  
76 variation, we focused on male body size because it classically determines competitiveness (and  
77 possibly mate attractiveness), longevity and hence potential number of matings during lifespan.  
78 In *V. canescens*, male size is a relevant trait; it is variable (Metzger, 2008) and may be a criterion  
79 of male quality used by females for mate choice when ejecting males at the beginning of mount  
80 (E.D. and B.C. pers. obs.). We considered potential sources of intra-individual variations in  
81 sexual behaviours by investigating effects of age and mating experience of males.

82 We also experimentally studied the impact of social environment variation on sexual  
83 behaviours through variation in operational sex ratio (OSR). This question makes sense because  
84 males and females encounter, and mate, in “rendez-vous” sites (e.g. fruits containing hosts),  
85 (Metzger, et al., 2010b). As a consequence, adult density and thus individual interactions are  
86 expected to vary, leading to different operational sex-ratio (OSR) in these sites. Because of the  
87 inverse sexual size dimorphism in *V. canescens*, this experiment allowed us to test the ‘agility  
88 hypothesis’ predicting that smaller males are superior in scramble competition and mate more  
89 frequently or rapidly than larger males (Andersson & Norberg, 1981; Blanckenhorn, 2005).  
90 This advantage for smaller males should be more acute under stronger male competition (i.e.  
91 more male-biased OSR).

92 At last, we investigated the effect of OSR on the intensity of sexual selection on male  
93 size, predicting a negative selection on this trait and an assortative pattern where smaller males  
94 mate with larger females (i.e. with higher egg load), and larger males with females  
95 independently of their size.

96

## 97 **Materials and methods**

### 98 Insect and rearing facilities

99 *Venturia canescens* is a koinobiont (i.e. hosts continue to develop after parasitization) and  
100 solitary (i.e. a single adult at most emerges from a given parasitized host) parasitoid wasp of a  
101 broad range of moth species (Salt, 1976). To access mating, males court with stereotyped

102 behaviours. Courtships are composed of five steps: localisation, orientation, positioning,  
103 mounting and mating. During orientation and positioning, the male whirrs its wings and swings  
104 sideways. Females may reject a male when it tries to mount or mate them, leading to a failure  
105 from the male point of view (van Santen & Schneider, 2002). Experiments and observations  
106 were conducted with sexual (parthenogenetic arrhenotokous) individuals, which have a  
107 haplodiploid sex determination, allowing us to quantify males' offspring by counting the  
108 number of daughters that come from fertilized eggs. This measure is a reliable proxy of male  
109 reproductive success as females do not control offspring sex-ratio (Metzger et al., 2008). In the  
110 following experiments, we quantified sexual behaviours by calculating: male latency to court a  
111 female, average courtship duration, latency to mate and to re-mate, and duration of each mating.

112 Wasps used in this study came from a lab rearing ( $24 \pm 1^\circ\text{C}$ ,  $50 \pm 10\%$  relative humidity, DL  
113 12:12). The strain was established from numerous individuals caught in the field during the  
114 whole summers 2017, 2018 and 2020 near Valence in an organic orchard (Drôme, France). As  
115 a host, we used the pyralid *Ephestia kuehniella*. All experiments were conducted under the same  
116 conditions between 10 am and 4 pm with individuals coming from different rearing boxes (to  
117 prevent from testing inbred individuals) and kept individually after emergence in plastic tubes,  
118 with food *ad libitum* (honey). Two hours before starting the observation, the food was removed  
119 to standardize the feeding status of individuals. In Experiments 1 and 2, individuals were  
120 anaesthetised with CO<sub>2</sub> and immediately placed in an arena (see Figure 1). Each arena was  
121 filmed using a Raspberry Camera HQ with Raspberry Pi 4, and videos were analysed with the  
122 BORIS software (Friard & Gamba, 2016).

### 123 Experiment 1: Mating system and male sexual behaviours

124 This experiment had two goals. First, we described male mating system. For that, we recorded  
125 sexual behaviours of single males in presence of three females. The presence of three females  
126 gave each male the opportunity to copulate several times during the observation period, and  
127 thus to test whether they are polygynous. Second, we estimated the costs of mating in terms of  
128 longevity and number of offspring produced by males.

129 To be able to identify the females a male mated with, each female was marked by a dot of water-  
130 paint (blue, green or orange) on the thorax. This procedure does not affect mating behaviour in  
131 *V. canescens* (Fauvergue et al., 2015). To avoid the establishment of owner *versus* intruder  
132 status, each anaesthetised wasp was confined (thus isolated from the other females) in a corner  
133 of an arena by a removable wall (Figure 1A). When all individuals were awake, we opened the

134 wall first for the 3 females (Figure 1B), allowing them to be acclimated to the experimental  
135 conditions. Thirty minutes later, we released the male, and recorded male sexual behaviours for  
136 90 minutes (Figure 1C).

137 To appraise male reproductive success, at the end of the observation, we gave each mated  
138 female the opportunity to oviposit in non-limited healthy hosts. We then calculated the  
139 proportion of daughters among the emerging parasitoids. We also quantified male longevity by  
140 keeping them individually in plastic tubes without food, and checking four times a day (at 8  
141 am, 12 am, 4 pm and 8 pm) whether they were still alive. Longevity was estimated from the  
142 median between the time found dead and the last time it was observed alive. Body size was  
143 considered as an inter-individual source of variation in sexual behaviours (courtship and  
144 mating). The size of individuals was approximated by the measure of their hind left tibia length  
145 (Pelosse et al., 2011). We used a dissecting microscope with a camera (moticam 1000, software  
146 Motic Image Plus 2.0). A total of 72 males were tested; due to technical issues, videos were  
147 incomplete for 13 males, explaining the sample size differences according to measured trait.

148

#### 149 Experiment 2: Intra-individual variations in sexual behaviours

150 We investigated whether age affects males' sexual behaviours (courtship and mating  
151 characteristics). For that, one virgin male and one virgin female were placed in the arena. Once  
152 awake, they were released from their closed space by opening the removable walls at the same  
153 time and sexual behaviours were observed and recorded for 45 minutes. A previous experiment  
154 showed that males live an average of 16 days under the same laboratory conditions (data not  
155 shown), so we considered ages: 1 day old ("Young") and 7 days old ("Old"). The virgin females  
156 were 1 day old. Size of males was measured as in Experiment 1. A total of 208 males were  
157 tested.

#### 158 Experiment 3: Test of "agility hypothesis", effects of OSR on sexual selection and assortative 159 mating patterns

160 This experiment aimed at testing i) the 'agility hypothesis', ii) the intensity and direction of  
161 sexual selection on size, expected to be negative under the 'agility hypothesis', iii) the existence  
162 of an assortative mating where small males mated with larger females, and if this mating pattern  
163 varies with the intensity of male-male competition. For this purpose, we created population  
164 cages with a fixed wasp density but different operational sex ratio (OSR), assuming that the



165 more male-biased the OSR, the more intense the male-male competition. In each cage, we  
166 observed matings and measured the size of mated partners and of all unmated individuals (see  
167 below) using two proxies of size: hind left tibia length (as in Experiments 1 and 2) and the  
168 length of the first segment of the abdomen.

169 Fifty randomly chosen individuals, one or two days old, were placed in a population cage  
170 (30\*30\*30 cm). In each cage, the OSR was 0.3, 0.5 or 0.7 corresponding to 35 females/15  
171 males, 25/25 or 15/35, respectively. We observed each cage for a maximum of 2 hours. We  
172 recorded the time between the start of the observation and the formation of the pairs, and  
173 removed them immediately from the cage. This procedure of removing the copulating pairs was  
174 repeated until 2 hours had elapsed or half of the possible matings had taken place (8 matings  
175 for OSR = 0.7 and 0.3, 13 matings for OSR = 0.5; this allowed the individuals still in the cage  
176 to have several choices for mating). Three replicates of each OSR condition were conducted.

#### 177 Data analysis

178 Experiment 1 showed that males are polygynous (see Results). Since only 4 males mated three  
179 times during the observation period, we grouped them with those that mated 2 times, and thus  
180 considered 3 categories of males for the statistical analyses: virgin, mated once and re-mated.

181 To appraise body size as an inter-individual source of variation in mating characteristics, we  
182 fitted different GLM. First, we tested the effect of male size i) on the probability to be mated at  
183 the end of the observation (GLM, binomial distribution, logit link), ii) on latency before first  
184 mating, and on first mating duration (GLM, Gamma distribution and inverse link). For these  
185 last two models, only mated individuals were analysed.

186 Then, we tested the effect of inter-individual variability of males on courtship characteristics  
187 (latency and average duration). As for mating, this variability was represented by male size  
188 differences and analysed by the means of GLM (Gamma distribution and inverse link).

189 We investigated the intra-individual variability in sexual behaviour by comparing males'  
190 characteristics of first and second matings (mating duration and latency before mating of re-  
191 mated males). We used a Wilcoxon test on paired data. Intra-individual variability of the  
192 courtship characteristics was analysed by comparing average duration of courtships and latency  
193 before the first courtship displayed by a given male when virgin and then mated.

194 Cost associated with mating in terms of longevity was analysed by the means of a GLM  
195 (Gamma distribution and inverse link) with the male reproductive status (3 modalities: virgin,

196 mated (one time) and re-mated), size and their interaction as explanatory variables. Cost in  
197 terms of sex ratio was analysed with a mixed GLM (binomial distribution, logit link) with  
198 mating rank (i.e. if the mating was the first or subsequent (as second and third matings were  
199 pooled)) as explanatory variable and male identity as random factor. For testing the intra-  
200 individual variability, we compared sex-ratio among re-mated males with a Wilcoxon test on  
201 paired data.

202 In Experiment 2, because of the variability between males in courtship occurrence, we first  
203 analysed the probability to court as a function of male age, size and their interaction (GLM  
204 binomial distribution, logit link). Among males that displayed courtships, we conducted the  
205 same analyses for the sexual behaviours (mating and courtship characteristics) as in Experiment  
206 1, but we added age as an explanatory variable together with its interaction with size. Male age  
207 was a qualitative variable, with two modalities (“Young” and “Old”).

208 For Experiment 3, we tested the ‘agility hypothesis’ by testing whether small males mated faster  
209 than large males, and whether this relationship between latency and male relative size depended  
210 on OSR (GLM Gamma distribution, inverse link; latency before mating as response variable,  
211 relative size, OSR (as factor) and their interaction as explanatory variables). Male relative size  
212 (MRS) was calculated as follows:  $MRS = \frac{x - \bar{x}}{\bar{x}}$ , where  $x$  is the size of the male and  $\bar{x}$  is the mean  
213 male size in the cage.

214 We estimated the intensity of sexual selection on female and male size for each OSR condition  
215 following the method presented in Arnold & Wade (Arnold & Wade, 1984) (see Appendix 1).

216 To assess the assortative mating pattern, we calculated the correlation between the two proxies  
217 of male size (tibia and abdomen sizes) to decide whether we should conduct the analysis on  
218 both or a single body measure (Spearman’s rank correlation). We then modelled assortative  
219 mating pattern by using mixed linear models (*lmer* function from *lme4* package, Bates et al.,  
220 2015) with male size as response variable, and female size and OSR (as fixed factors) as  
221 explanatory variables; cage (i.e. population cage) was included as a random factor. As male and  
222 female sizes could be both response variables, we swapped the role of the variables male size  
223 and female size in our previous model. Conclusions are qualitatively the same, we present only  
224 results with male size as response variable.

225 We presented the results from the simplest model selected after a backward procedure  
226 (Crawley, 2012). We accompanied results of the models with effect sizes (*effectsize* package,

227 Ben-Shachar et al., 2021). Effect sizes are presented with their 95% confidence interval.  
228 Analyses were performed with R (R Core Team, 2020).

229

## 230 **Results**

### 231 Experiment 1: Mating system and male sexual behaviours

232 Our results show that *Venturia canescens* males are polygynous. Fifty percent of the males  
233 mated, and 52.78% of these mated males re-mated at least once during the observation period  
234 of 1h30 (Figure 2). Smaller males tended to have a higher probability to be mated at the end of  
235 the observation ( $\chi^2 = 2.987$ ,  $df = 1$ ,  $p = 0.08$ ; medium effect size Hedges'  $g = 0.40$ ,  $IC = [-0.06,$   
236  $0.86]$ ), suggesting that body size is likely an inter-individual source of variation in mating  
237 probability. Nevertheless, among mated males, size did not influence latency before mating and  
238 mating duration (latency:  $F = 0.02$ , 1 and 32  $df$ ,  $p = 0.88$ ; duration:  $F = 0.81$ , 1 and 32  $df$ ,  $p =$   
239  $0.37$ ).

240 There is no evidence that size is a source of inter-individual variation in courtship  
241 characteristics. We did not detect any effect of male size on the latency before the first courtship  
242 ( $F = 0.426$ ,  $df = 1$  and 63,  $p = 0.517$ ; 8 over 72 males displaying no courtship were excluded  
243 from the analysis). The average duration of courtship of virgin males was independent of their  
244 size ( $F = 1.75$ , 1 and 54  $df$ ,  $p = 0.19$ ).

245 Analyses performed among the mated males suggested intra-individual variations in the  
246 average courtship duration according to mating experience. The average courtship duration  
247 decreased after mating (Figure 3A,  $6.0 (\pm 3.0 \text{ SE})$  sec when virgin *versus*  $4.0 (\pm 1.0)$  sec when  
248 mated) ( $N = 29$ ,  $V = 373$ ,  $p = 0.0004$ ; very large effect size Glass'  $\delta = 1.78$ ,  $IC = [0.63,$   
249  $2.90]$ ). On the contrary, latency before courtship did not change with mating experience ( $N =$   
250  $32$ ,  $V = 246$ ,  $p = 0.747$ ).

251 Among re-mated males ( $N=19$ ), we detected intra-individual variation in mating duration. The  
252 second mating was longer than the first one (Figure 3B,  $V = 0$ ,  $p = 0.0007$ ,  $N = 15$ ; very large  
253 effect size Hedges'  $g = -1.64$ ,  $IC = [-2.45, -0.81]$ ). The conclusion was similar when we included  
254 two extremes values of mating duration (Appendix 2). There was no difference in mating  
255 latency ( $N = 19$ ,  $V = 60$ ,  $p = 0.28$ ).

256 There was a tendency that matings were costly in terms of longevity. Virgin males lived 21%  
257 longer than mated males in average (Figure 4, median: 3.71 days for mated versus 4.46 days

258 for virgin males;  $F = 3.23$ ,  $df = 1$  and  $65$ ,  $p = 0.077$ ). This was confirmed by large effect size  
259 (Hedges'  $g = -0.65$ ,  $IC = [-1.13, -0.16]$ ). Nevertheless, there was no difference in longevity  
260 within mated males (median = 3.71 days is identical for mated and re-mated). Irrespective of  
261 the mating status, longevity was also affected by the male size; larger males live longer ( $F =$   
262  $30.81$ ,  $df = 1$  and  $65$ ,  $p < 0.0001$ ).

263 The reproductive success (offspring sex-ratio, SR) was not affected by the rank of mating when  
264 considering all mated males (first mating: mean SR =  $0.52 \pm 0.34$ ; re-mating: mean SR =  
265  $0.75 \pm 0.39$ ;  $\chi^2 = 0.784$ ,  $df = 1$ ,  $p = 0.376$ ). We did not detect intra-individual variation of SR,  
266 when comparing SR from the first and second matings within re-mated males (first mating:  
267 mean SR =  $0.55 \pm 0.32$ , second mating: mean SR =  $0.59 \pm 0.32$ ; Wilcoxon paired test,  $V = 62.5$ ,  
268  $p = 0.796$ ,  $N = 17$ ).

269

#### 270 Experiment 2: Intra-individual variations in sexual behaviours

271 Old males had a higher probability to court than young males (59.22% of old males *versus*  
272 31.43% of young males courted the females,  $\chi^2 = 16.44$ ,  $df = 1$ ,  $p < 0.0001$ ; medium effect size:  
273 Odd Ratio = 3.15,  $IC = [1.73, 5.83]$ ). Nevertheless, male age influenced neither courtship  
274 characteristics: average courtship duration ( $F = 1.73$ ,  $df = 1$  and  $91$ ,  $p = 0.192$ ) and latency  
275 before the first courtship ( $F = 0.068$ ,  $df = 1$  and  $92$ ,  $p = 0.79$ ), nor mating: the mating probability  
276 ( $\chi^2 = 0.262$ ,  $df = 1$ ,  $p = 0.61$ ), mating latency ( $F = 1.33$ ,  $df = 1$  and  $29$ ,  $p = 0.26$ ), mating duration  
277 ( $F = 1.73$ ,  $df = 1$  and  $29$ ,  $p = 0.199$ ). Male size did not affect any of the response variables in  
278 this experiment.

279

#### 280 Experiment 3: Test of “agility hypothesis”, effects of OSR on sexual selection and assortative 281 mating patterns

282 *Venturia canescens* presented an inverse sexual dimorphism in size: males are smaller than  
283 females irrespective of the proxy used to estimate the size (mean tibia length for males =  $1.58$   
284  $\pm 0.16$  mm; for females =  $1.73 \pm 0.13$  mm;  $t = -10.02$ ,  $df = 354$ ,  $p < 0.0001$ ; mean length of the  
285 first segment abdomen for males =  $1.06 \pm 0.10$  mm, for females =  $1.29 \pm 0.10$  mm,  $t = -21.06$ ,  
286  $df = 354$ ,  $p < 0.0001$ ). The two proxies of body size were positively correlated both in males and  
287 in females (in males:  $\rho = 0.89$ , in females:  $\rho = 0.86$ ). We therefore present the results concerning  
288 the length of the tibia (results with the first segment of the abdomen are in Appendix 3 and 4).

289 The latency before mating was influenced neither by relative size of males (MRS) in a cage (F  
290 = 0.719, df = 1 and 83, p = 0.399) nor by the male-male competition (i.e. OSR) (F = 2.546, df  
291 = 1 and 82, p = 0.085, Figure 6). This indicated that our data did not support the agility  
292 hypothesis. This conclusion is confirmed by the fact that the intensity of sexual selection,  $I$ , on  
293 size remained weak (and non-significantly different from zero) whatever the sex and the OSR  
294 conditions (Table 1). When conducting analyses for each cage separately, we detected an  
295 intensity of sexual selection different from 0 only in the cages 1, 2 and 8 for males, and in cage  
296 8 for females. The direction of sexual selection was either weakly negative or positive (Table  
297 1). These values of  $I$  also indicate that there is no evidence of any relationship between male  
298 body size and the mating probability.

299 We did not detect any clear pattern of assortative mating. Indeed, there was no correlation  
300 between male and female size within pairs formed in the cages (Figure 5,  $\chi^2 = 0.03$ , df = 1, p =  
301 0.86) whatever the OSR (interaction between female size and OSR:  $\chi^2 = 0.933$ , df = 2, p =  
302 0.627). The conclusions were the same when we analysed the female size as response variable  
303 and male size as explanatory variable (male size effect:  $\chi^2 = 0.142$ , df = 1, p = 0.707, interaction  
304 between male size and OSR:  $\chi^2 = 0.234$ , df = 2, p = 0.89).

305

## 306 **Discussion**

307 Our results provide a more detailed picture of the mating system in *Venturia canescens*  
308 males, of the variations in sexual behaviours at inter- and intra-individual levels, and their  
309 consequences in terms of reproductive costs. At last, we assess the intensity and direction of  
310 sexual selection on body size as a potential trait involved in mating.

311 Males are polygynous (this study) and females are monandrous (Collet et al., 2020).  
312 Such a polygynous mating system implies that some males have no access to reproduction while  
313 others are more successful, opening opportunities for assessing reproductive costs. A cost in  
314 terms of realised fecundity (i.e. a decrease of the offspring number per reproductive event  
315 during lifetime) is often described in non-social insects (Walker, 1980), but we did not detect  
316 such a cost in male *V. canescens*, at least in the case of two matings close together in time.  
317 Indeed, the offspring sex-ratio did not change with the rank of successive matings in *V.*  
318 *canescens*, suggesting that sperm depletion did not occur, while it is observed in males of  
319 several parasitoid species (Damiens & Boivin, 2006). However, we cannot rule out that sperm  
320 depletion could have occurred after a greater number of matings over a short period of time,

321 preventing *V. canescens* males from replenishing their sperm stock between matings. This is  
322 the case in *Trichogramma evanescens* in which males transfer 50% of their sperm after 8  
323 matings (Damiens & Boivin, 2005). Alternatively, in our experiments a weak sperm depletion  
324 could have occurred, compensated for by a longer mating duration (potentially associated with  
325 a greater transfer of spermatozooids) as observed in the second mating (Experiment 1). This  
326 positive correlation between copulation duration and quantity of transferred sperm, is far from  
327 being ubiquitous in insects (Weggelaar et al., 2019) but was described, for instance, in the  
328 bushcricket *Requena verticalis* (Simmons & Achmann, 2000). Regarding longevity, we  
329 observed a cost incurred by mated males that was not accentuated with multiple matings. This  
330 cost is classically observed in male arthropods (Scharf et al., 2013). In the Australian tettigoniid  
331 *Kawanaphila nartee*, breeding males live 6% shorter than non-breeding males, and this  
332 difference increases when the resources are low, leading to a 48% loss in longevity linked to  
333 breeding (Simmons & Kvarnemo, 2006). Proximate factors responsible for the longevity  
334 decrease are diverse. For instance, in our laboratory experiments, we could not assess the costs  
335 of mate-finding and male-male competition (at least on the two first experiments). Therefore,  
336 we probably under-estimated the cost of reproduction, since flight is known to be highly energy  
337 demanding in *V. canescens* (Amat et al., 2012) and that male-male interactions could reduce  
338 the lifespan (B.C. data not shown). A complete picture of reproductive costs in males would  
339 require further investigations.

340         Age is often reported as an intra-individual source of variation in sexual behaviours. For  
341 instance, females of the butterfly *Bicyclus anynana* prefer to mate with mid-aged males, so  
342 young and old males have a lower mating success (Nieberding et al., 2012). Our experiments  
343 suggested that courting probability increased with male age while other courtship  
344 characteristics were not affected by age. These results could be explained by a physiological  
345 change with age (e.g. hormonal modification or motivation). Facing a reduced residual lifespan,  
346 males would be more active in courtship. Another explanation for the higher courting  
347 probability in older males would be that their courtships are of lower quality. They therefore  
348 would need more courtships to seduce a female and achieve mating. At last, this behavioural  
349 change could be due to a greater experience in old males as shown in *Drosophila melanogaster*  
350 male (Balaban-Feld & Valone, 2017). However, this last explanation is unlikely in our study  
351 since old and young males had the same mating experience when tested in Experiment 2.  
352 Nevertheless, we can imagine that male experience during its life impacts its motivation to  
353 mate, as we found that courtship duration decreased after the first mating (Experiment 1).

354 Motivation thus may vary with age according to males' reproductive history. Surprisingly, this  
355 effect of male age on courtship behaviours did not result in difference of mating probability  
356 between young and old males. So, age was not a relevant cue for the choice of the sexual partner  
357 by the females or did not confer any advantage for the males. Unfortunately, we did not compare  
358 the offspring production of young and old males. This precludes to firmly conclude about an  
359 effect of ageing in males.

360 Because of the inverse sexual dimorphism in size, we predicted that male size was under  
361 negative selection and expected that smaller males were superior in scramble competition for  
362 access to females ('agility hypothesis', Andersson & Norberg, 1981). In experiment 1, in  
363 absence of competition, we detected a weak evidence of greater mating probability in small  
364 males suggesting that agility hypothesis is potentially supported in *V. canescens*. However, our  
365 results in population cages clearly invalidated this hypothesis: smaller males did not have  
366 shorter latency before mating even under strong intra-sexual competition (i.e. when OSR is  
367 male-biased). This conclusion is also corroborated by the fact that our measures of sexual  
368 selection intensity and direction indicated that size was not the target of sexual selection. We  
369 also expected that operational sex-ratio (OSR) influenced assortative mating patterns.  
370 However, we only observed random pairing patterns according to size whatever the intensity  
371 of male-male competition. This absence of assortative mating by size is coherent with our  
372 results concerning the weak sexual selection on size. The removal of mating pairs from the  
373 cages could have prevented us from observing skewed mating success according to body size,  
374 for instance if some males mated several times. To reinforce our conclusions, an experiment in  
375 which individuals are individually marked and thus identified in each pair would be relevant to  
376 follow potential multiple matings.

377 Our experimental results confirmed that males are polygynous but did not allow us to  
378 validate the hypothesis that a competitive advantage of the smaller males (agility hypothesis)  
379 is a determinant of scramble competition polygyny (as described in Thornhill & Alcock 1983)  
380 in population of *V. canescens*. It would be relevant to conduct complementary field experiment  
381 - with the difficulties associated with monitoring minute insects - to test whether the rank of  
382 males' arrival at the 'rendez-vous' sites (e.g. fruits) could be determinant of mating opportunity.  
383 The males that are the fastest to locate odour combination of females and their hosts, and the  
384 fastest to reach these sites should have an advantage in accessing to virgin females.

385

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508

509

510 **Figures**

511

512

513 FIGURE 1: Experimental arena seen from above for Experiment 1, with removable walls  
514 represented in green. A: each anaesthetised individual is placed in a closed corner of the arena;  
515 B: then the 3 awake females are released in the arena by opening the walls (red arrows) but the  
516 male is still confined to his corner during 30 minutes; C: finally, the male is released by opening  
517 the wall (red arrow) and the observation begins for 90 minutes.

518 FIGURE 2: Distribution of the number of matings per male (Experiment 1).

519 FIGURE 3: Difference in sexual behaviours when a male is virgin versus mated. A: difference  
520 in mean courtship duration before and after the first mating. B: difference in mating duration  
521 between first and second matings for re-mated males. Two extremes values (mating durations  
522 = 241.5 and 495 sec.) were removed from the dataset (Experiment 1).

523 FIGURE 4: Relationship between longevity, male status and size (Experiment 1); lines  
524 represent the predictions of the selected statistical model; shaded areas represent the confidence  
525 interval of prediction.

526 FIGURE 5: Relationships between size of sexual partners when mated under different  
527 conditions of operational sex ratio (OSR) (Experiment 3).

528 FIGURE 6: Relationship between latencies before mating and OSR. Kaplan-Meier plot  
529 showing the probability to mate according to time (Experiment 3). Shaded areas represent  
530 confidence interval of prediction.

531

532 Figure 1

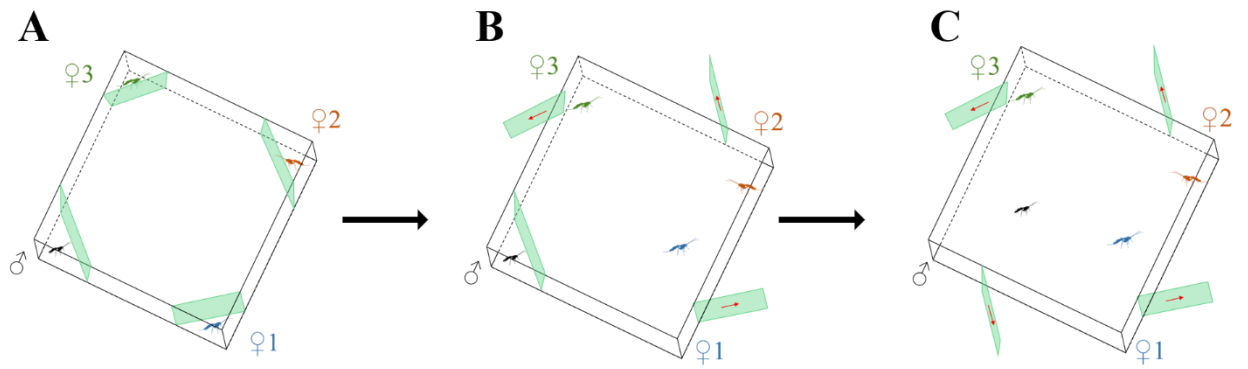
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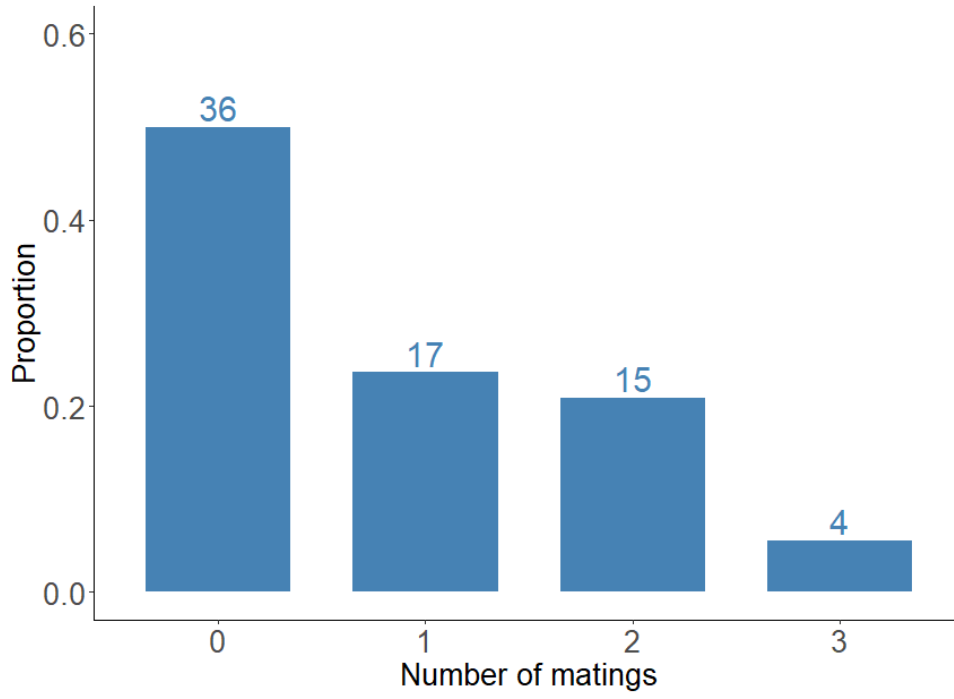
538 Figure 2

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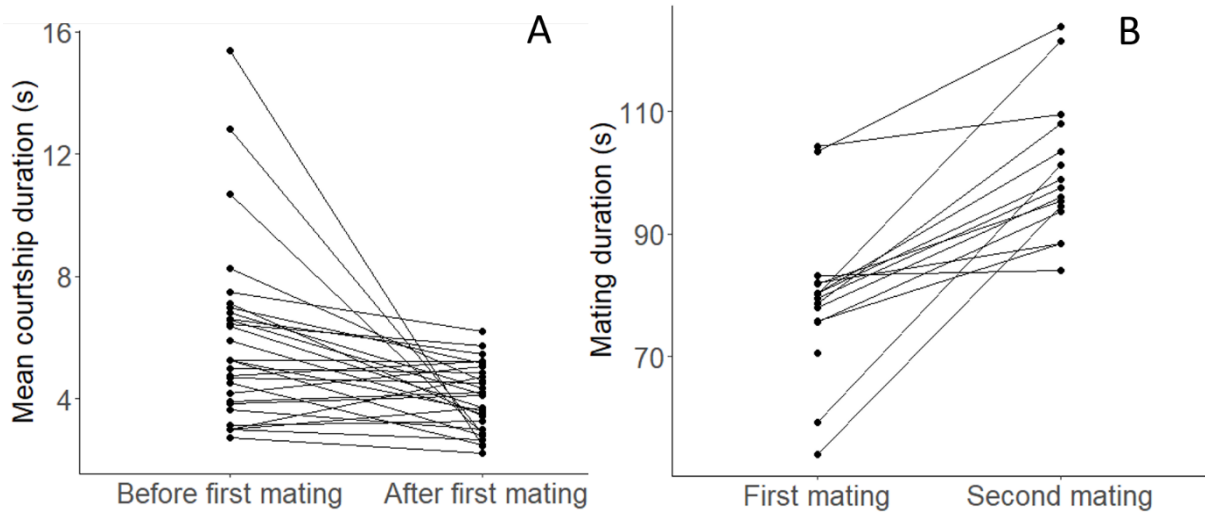
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543 Figure 3

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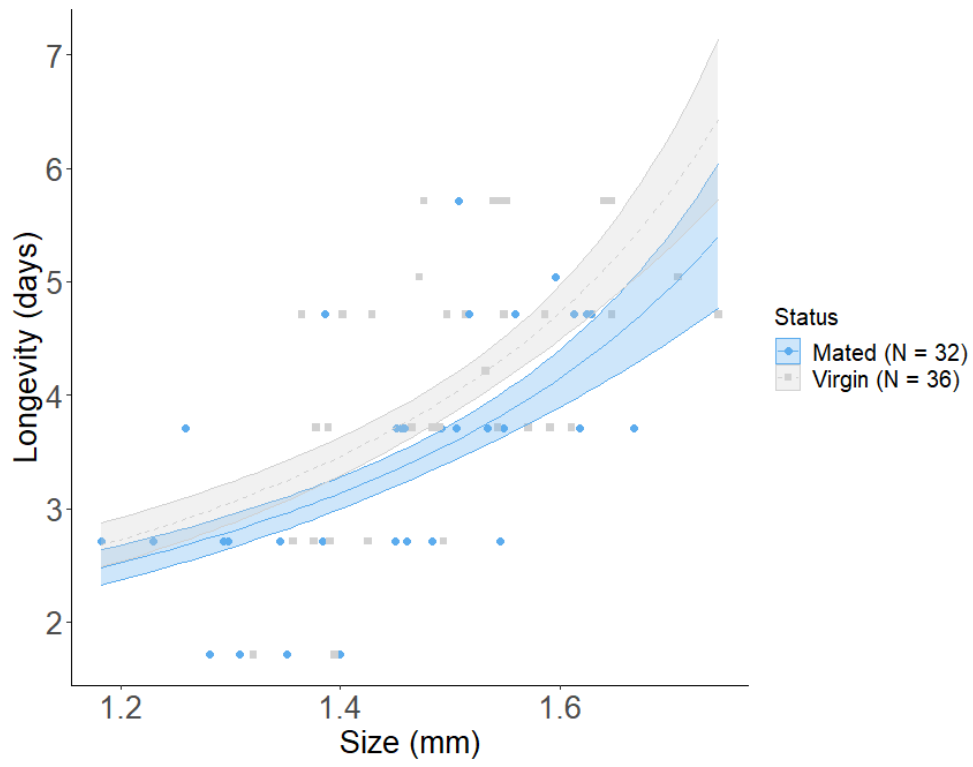
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547 Figure 4

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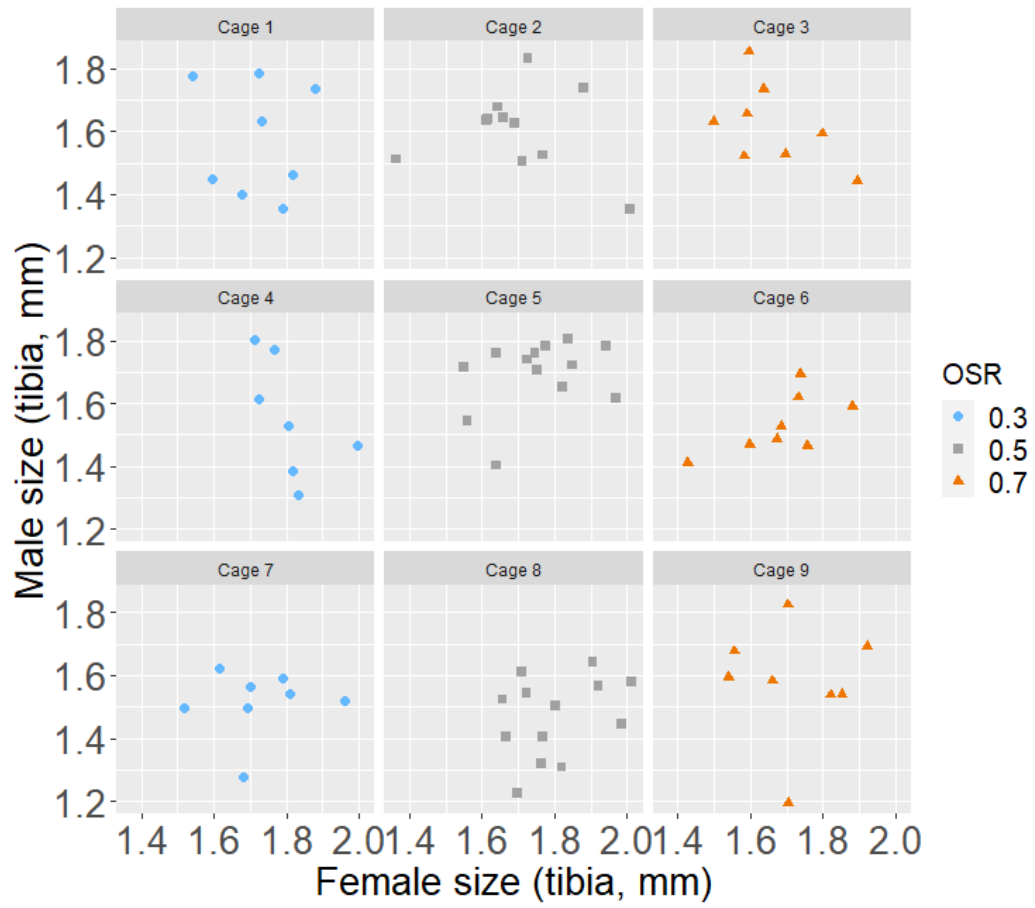


551 Figure 5

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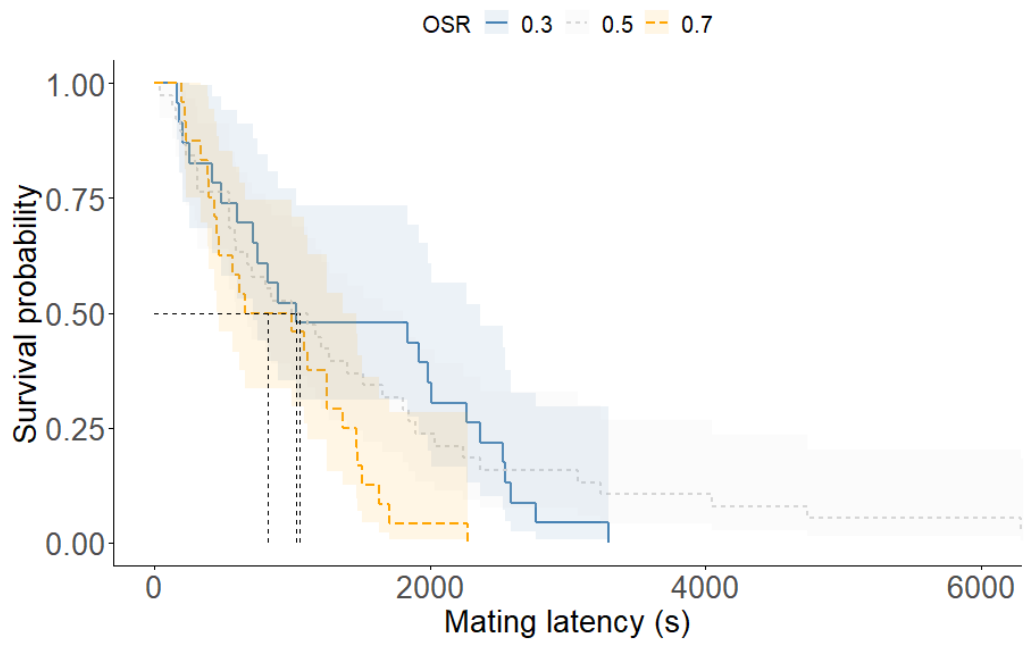


555 Figure 6

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558



559 **Tables**

560

561 Table 1: Sexual selection opportunity, I, on size for males and females in each OSR and Cage. p-values  
 562 come from permutation tests. OSR: Operational sex-ratio in population cage (Experiment 3)

563

564

OSR	Males		Females		Cage	Males		Females	
	I	p-value	I	p-value		I	p-value	I	p-value
<b>0.3</b>	0.1088	0.469	0.0601	0.741	<b>1</b>	<b>0.4697</b>	<b>0.045</b>	-0.0038	0.990
					<b>4</b>	0.0206	0.939	0.4924	0.148
					<b>7</b>	-0.3753	0.109	-0.2195	0.484
<b>0.5</b>	0.0269	0.814	0.1077	0.348	<b>2</b>	<b>0.4113</b>	<b>0.047</b>	-0.2329	0.270
					<b>5</b>	0.3347	0.084	0.0152	0.938
					<b>8</b>	<b>-0.5573</b>	<b>0.001</b>	<b>0.4561</b>	<b>0.011</b>
<b>0.7</b>	0.1105	0.538	-0.0495	0.747	<b>3</b>	0.4503	0.148	-0.1899	0.440
					<b>6</b>	-0.1274	0.687	0.0133	0.962
					<b>9</b>	0.0461	0.888	-0.0032	0.991

565

566 **Appendices**

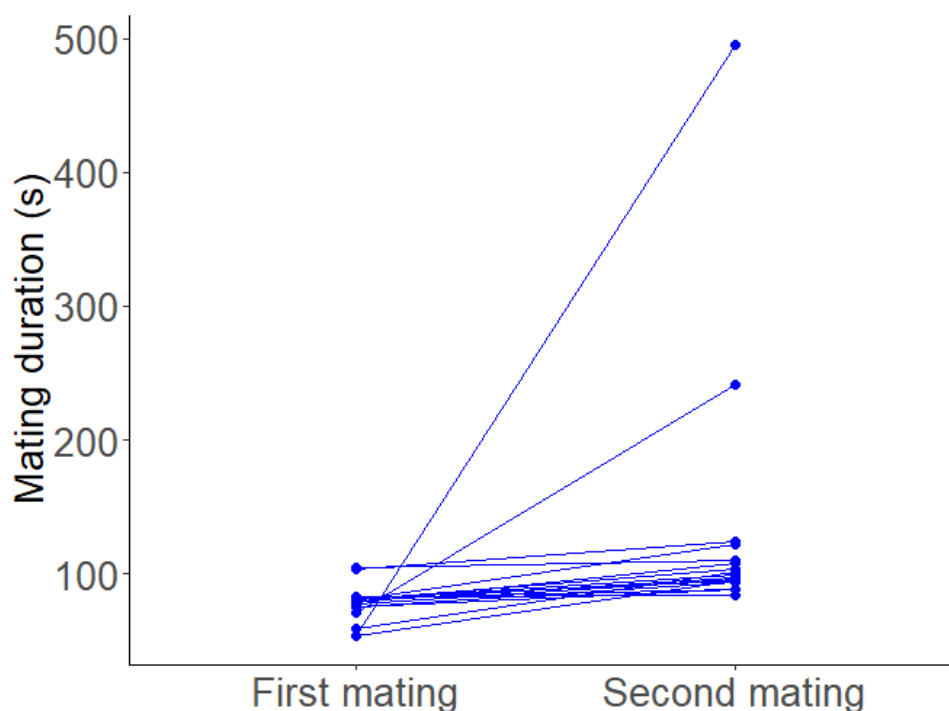
567 **Appendix 1 - Estimation of the intensity of sexual selection on female and male size for each**  
568 **OSR condition**

569 The intensity of sexual selection on size was calculated as:  $I_i = \frac{S_i}{\sqrt{\text{var}(z)}}$  where  $S_i$  is the selection  
570 differential on size and  $\text{var}(z)$  is the overall variance in size. Selection differential is defined as:  
571  $S_i = \bar{z}_i - \bar{z}$ , where  $\bar{z}_i$  is the mean size of paired individuals, i.e. the mean size after selection, and  
572  $\bar{z}$  is the mean size of all the individuals, i.e. the mean size before selection. We tested the null  
573 hypothesis,  $I = 0$  through a randomization test, as described in Sokal & Rolf (Sokal & Rolf,  
574 1995) with 10,000 permutations.

575

576 **Appendix 2 - Difference of mating duration between first and second matings in re-mated**  
577 **males when considering the two extremes values**

578 In Experiment 1, two extremes mating durations were recorded (241.5 and 495 sec). They were  
579 withdrawn from the dataset. We present here the same figure as Figure 3B but including these  
580 two extremes values. Conclusions are qualitatively unchanged ( $V = 0$ ,  $p = 0.0003$ ,  $N = 17$ ;  
581 medium effect size Glass' delta = -0.54, IC = [-1.05, -0.02])).



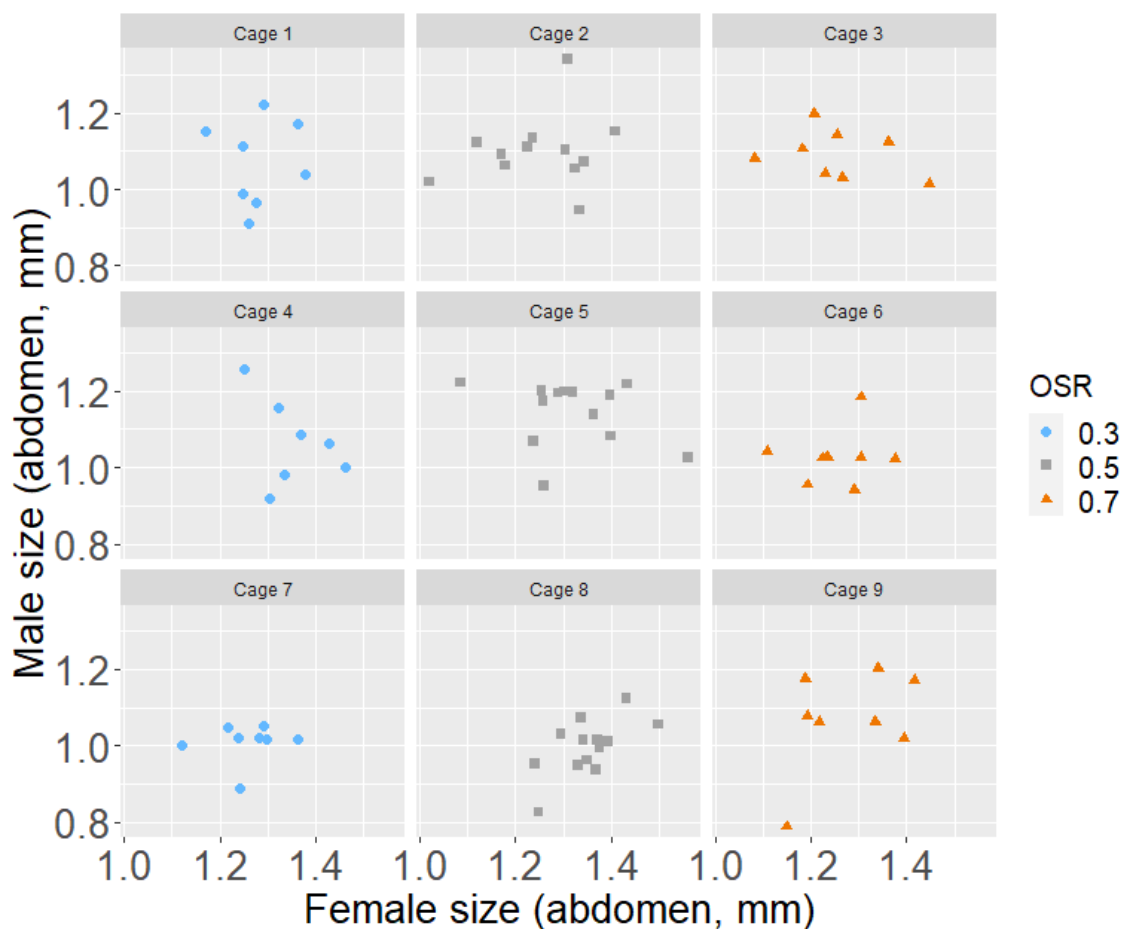
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583 *Figure Appendix 2: Difference in mating duration between first and second matings for re-*  
584 *mated males, including two extremes values of mating duration.*

585

586 **Appendix 3 - Do males and females *Venturia canescens* mate assortatively according to**  
 587 **their size? Is this pattern influenced by OSR?**

588 The results presented below were obtained with the measure of the first segment of the abdomen  
 589 as size proxy. There was no evidence of relation between male and female sizes ( $\chi^2 = 0.347$ ,  $df$   
 590  $= 1$ ,  $p = 0.56$ ), confirming that there was no assortative mating by size in *V. canescens*. The  
 591 effect of interaction between female size and OSR on male size was not significant ( $\chi^2 = 0.536$ ,  
 592  $df = 2$ ,  $p = 0.77$ ) irrespective of the OSR. The same conclusions were obtained when we  
 593 analysed the female size as response variable and male size as explanatory variable (male size  
 594 effect:  $\chi^2 = 0.27$ ,  $df = 1$ ,  $p = 0.60$ , interaction between male size and OSR:  $\chi^2 = 0.57$ ,  $df = 2$ ,  $p$   
 595  $= 0.75$ ).



596  
 597 *Figure Appendix 3: Assortative mating pattern between male and female size (length of the first*  
 598 *segment abdomen used as size proxy) and effect of OSR on this pattern.*

599  
 600 **Appendix 4- Is the opportunity for sexual selection on body size influenced by OSR? Study**  
 601 **using the measure of the first segment of the abdomen as male size proxy.**

602 Under the different conditions of OSR, there was no evidence allowing to conclude that sexual  
 603 selection acts on body size in males and females *V. canescens*. The intensity of sexual selection  
 604 was not different from 0 in the different population cages analysed separately, except in cages  
 605 1 and 8 for males, and in cage 8 for females (Table Appendix 4).

606 *Table Appendix 4: Sexual selection intensity, I, on size for males and females in each OSR and*  
 607 *Cage. p-values come from permutation tests. OSR = operational sex-ratio in population cage*  
 608 *(Experiment 3).*

OSR	Males		Females		Cage	Males		Females	
	I	p-value	I	P-value		I	P-value	I	p-value
<b>0.3</b>	0.1232	0.4071	-0.0127	0.9439	<b>1</b>	<b>0.5128</b>	<b>0.0253</b>	-0.0089	0.9743
					<b>4</b>	0.0610	0.8318	0.4884	0.1494
					<b>7</b>	-0.4270	0.0688	-0.4231	0.1727
<b>0.5</b>	0.0766	0.5011	0.1116	0.3382	<b>2</b>	0.3350	0.1141	-0.3279	0.1157
					<b>5</b>	0.3452	0.0706	0.1119	0.5705
					<b>8</b>	<b>-0.4982</b>	<b>0.0061</b>	<b>0.4879</b>	<b>0.0075</b>
<b>0.7</b>	0.0811	0.6489	-0.0326	0.8263	<b>3</b>	0.4889	0.1121	-0.0777	0.7569
					<b>6</b>	-0.1502	0.6400	0.1033	0.7831
					<b>9</b>	-0.0221	0.9448	-0.1965	0.4234

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