

1 Estimation of the propensity for sexual selection in a
2 cyclical parthenogen

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17 Short title: Sexual selection in *Daphnia*

18 Abstract

19 Cyclical parthenogenesis is a widespread reproductive strategy in which organisms go through one or
20 multiple rounds of clonal reproduction before sexual reproduction. In populations of the planktonic
21 cladoceran *Daphnia magna* sexual reproduction is typically less common than parthenogenesis and
22 therefore hardly studied. We studied the sexual process and its relation to sexual selection in *Daphnia*
23 rockpool populations, where sex is common throughout the summer, by observing natural mating in
24 these shallow habitats. While microsatellite markers revealed no evidence for disassortative mating
25 and thus, inbreeding avoidance, body length and infection status revealed assortative mating,
26 suggesting sexual selection to act. In cases where two males mated with a single female, larger male
27 remained longer, possibly giving them an advantage in sperm competition. Indirect evidence points at
28 the brood pouch as the likely site of fertilization and thus, sperm competition. Sperm length was as
29 variable within ejaculates as it was among males from different populations. Our data give firm
30 evidence that sexual selection is present in this species and that it likely manifests itself by a
31 combination of female choice and male - male competition.

32 Introduction

33 Sexual selection operates on the ability for fertilization and operates to varying degrees whenever
34 sexual reproduction occurs (Andersson 1994; Shuker 2010; Clutton-Brock 2017). Under sexual
35 selection, the traits that generally evolve jointly, but separately in each sex, are those related to the
36 choice of females for males or sperm and traits that allow males to compete with each other, either
37 directly or when facing female choice. On the one hand, females choose based on characteristics such
38 as male vigor to maximize the success of their progeny, such as size, ornament, and infection status.
39 On the other hand, males are likely to have higher mating success when they possess better condition-
40 dependent traits, such as mate searching intensity, fighting ability, sperm quality or some types of
41 exaggerated morphological characters (Andersson 1994; Morehouse 2014; Kaldun and Otti 2016;
42 Houslay et al. 2017). Sexual selection has thus the potential to reduce mutation load and has, for this
43 reason, been invoked as one of the forces maintaining costly sexual reproduction (Whitlock and
44 Agrawal 2009; Lumley et al. 2015). In fact, the potential evolutionary benefits of sexual selection
45 probably explain why organisms with sole asexual reproduction are extremely rare.

46 Organisms where sexual reproduction alternates with asexual reproduction are said to
47 reproduce by cyclical parthenogenesis. Examples are found in many taxa, such as aphids, stick insects,
48 rotifers, parasitic nematodes of human, and even vertebrates (Hand 1991; Lampert 2009). In
49 freshwater cladocerans from the genus *Daphnia*, periods of asexual reproduction are punctuated by
50 events of sexual reproduction. These model crustaceans play an important role in our understanding of
51 fundamental biological concepts such as the capacity of immune cells to engulf foreign antigens
52 (Metschnikoff 1884), the definition of germ line and soma (Weismann 1893), phenotypic plasticity
53 (Woltereck 1909), the capacity of natural populations to genetically adapt to anthropogenic stressors
54 (Jansen et al. 2011), parasite local adaptation (Reger et al. 2018), host-parasite coevolution
55 (Decaestecker et al. 2007; Ebert et al. 2016), phenotypically plastic response to biotic and abiotic
56 stressors (Tollrian and Heibl 2004; Cavalheri et al. 2019), and even the consequences of climate
57 change on animal populations (George et al. 1990; Carter et al. 2017). The overwhelming majority of
58 studies using *Daphnia* focus on the asexual mode of reproduction. We argue that understanding sexual

59 reproduction in cyclical parthenogenetic species offers the opportunity to study the evolution of traits
60 presumably under sexual selection after periods of clonal reproduction.

61 The biology of sexual reproduction in the genus *Daphnia*, including mating and fertilization,
62 is largely unexplored, and evidence for sexual selection was to our knowledge never presented. The
63 few studies addressing aspects of sexual reproduction in *Daphnia* are limited laboratory conditions
64 and conducted with different species with seemingly different ecologies (Brewer 1998; Winsor and
65 Innes 2002; Wuerz et al. 2017). In *Daphnia*, sexual reproduction is linked to dormancy as the two
66 sexual eggs are protected by a hard melanized case, called ephippium, that allows embryos in
67 developmental arrest to survive summer draughts and freezing conditions in winter. Sexual
68 reproduction is key to the long-term persistence of *Daphnia* populations in unstable environments,
69 creating genetically diverse egg-banks from which future populations are established. The frequency
70 of sexual reproduction correlates with habitat instability at a continental scale (Roulin et al. 2013).

71 Sex is environmentally determined in *Daphnia* (Hobæk and Larsson 1990) and males and
72 females are believed to be genetically identical (Hebert and Ward 1972). Males and females have
73 different morphology and swimming behavior corresponding to their respective role in reproduction
74 (Brewer 1998; Ebert 2005; Wuerz et al. 2017). Differences between sexes is traditionally based on
75 Darwin's theory of sexual selection (Darwin 1871; Shine 1979; Clutton-Brock 2017) or on selection
76 by intraspecific niche divergence (Cox and Calsbeek 2010; Law and Mehta 2018). As there is no
77 indication that niches diverge between sexes in *Daphnia*, sexual selection may be the main factor
78 acting on sexually dimorphic traits. However, little is known about mating in *Daphnia*. Unlike
79 copepods (Lonsdale et al. 1998), and even though *Daphnia* respond behaviorally and phenotypically
80 to several chemical cues such as fish kairomones (Hahn et al. 2019), there is no evidence for sex
81 pheromones that males use to find their mate (Crease and Hebert 1983; Winsor and Innes 2002). Yet,
82 male swimming behavior seems to be optimized to find an appropriate mate (Brewer 1998) and
83 mating is not random as males capture sexually active females (i.e. females carrying ephippial egg
84 cases) more often than they capture other males or asexual females (Brewer 1998; Winsor and Innes
85 2002). Thus, it seems plausible that mating couples are formed based on certain criteria, possibly

86 reflecting individual quality. Given the tremendous variation in mating duration among *Daphnia*
87 species (from few seconds up to a day (Forró 1997)), it is difficult to generalize conclusions from one
88 species to another, probably because the genus is believed to be older than 140 million years (Cornetti
89 et al. 2019), predating the placental mammal diversification (Springer et al. 2003)). Here, we
90 investigated the interactions of males and females from mate finding to the release of the fertilized
91 resting egg in natural populations of *Daphnia magna*. Our data provides evidence that sexual selection
92 is present in this species and that it likely manifests itself by a combination of female choice and male
93 - male competition.

94 **Materials and methods**

95 **Study area.** We studied *Daphnia magna* in a metapopulation on the coast of the Baltic Sea in South-
96 Western Finland, near Tvärminne Zoological Station (59°50' N, 23°15'E). The rockpools in this
97 metapopulation are small (average volume about 300 L) and shallow (10 to 60 cm deep) (Altermatt
98 and Ebert 2010), allowing easy access to every part of the habitat. About 40 % of the rockpools in this
99 area are inhabited with at least one *Daphnia* species (Pajunen 1986). Our field work was performed
100 over the course of four summers (2003, 2009, 2010 and 2011) and included 33 rockpool populations
101 (See supplementary material 1 for further information).

102 **Data collection.** To estimate the sex-ratio in populations, we randomly collected planktonic *D. magna*
103 with handheld nets (mesh size 0.3 mm) or by sampling 1 L of water. The shallow pools allowed us to
104 search and collect mating pairs with wide-mouth pipettes. The mating pairs were then kept separately
105 in 25-mL jars and observed in 1-minute intervals. We recorded the number of males concomitantly
106 attached to the female, the time period until a male detached from the female and the order of
107 detachment when there was more than one male. Using a field dissecting scope, we observed the
108 females and recorded the time post mating at which females laid the sexual eggs in the brood pouch,
109 which by this time already assumes the typical shape of a resting egg case. Females were then kept in
110 the jars until they dropped the resting egg case. We measured body- and spine-length before checking
111 for parasites under a microscope or storing them in ethanol at -20 °C.

112 The studied parasite was *Hamiltosporidium tvarminnensis*, a microsporidium commonly found in the
113 studied metapopulation but not only (Haag et al. 2011; Goren and Ben-Ami 2013). It is found to infect
114 several *Daphnia* species but its success and its pathogenicity are very host specific (Vizoso and Ebert
115 2005; Sheikh-Jabbari et al. 2014; Urca and Ben-Ami 2018; Orlansky and Ben-Ami 2019). It has a
116 mixed mode of transmission, and can be transmitted vertically or horizontally, when spores are
117 released from the decaying cadaver (Lass and Ebert 2006).

118 To measure sperm length, we exposed males to 1 % nicotine solution, which stimulates muscle
119 contractions and results in a release of sperm (as in Duneau et al. 2012). As only mature sperm are in
120 the testicular lumen (p. 11 in Wingstrand, 1978; p 277 in Zaffagnini, 1987), this method is better than
121 crushing the males where immature sperms of various length could be found. We then took pictures
122 with a camera mounted on a microscope (magnification x200) and measured the longest length of
123 several sperms in the sample with ImageJ (version 1.50i).

124 **Genotyping.** To genotype individuals, we homogenized them individually in 100 μ l of Chelex solution
125 and used a Chelex DNA extraction protocol (Walsh et al. 1991), before performing a PCR on *Daphnia*
126 microsatellite markers (see details in supplementary material 1 – section 5). PCR reactions of 5 μ L
127 were set up with the following cycling conditions: 95 °C for 15 min, followed by 30 cycles of 94 °C
128 for 30 s, 60 °C for 1.5 min, 72 °C for 1.5 min and 10 cycles of 94 °C for 30 s, 47 °C for 1.5 min, 72 °C
129 for 1.5 min, and a final elongation step of 72 °C for 10 min. Genotyping was done on an *AB 3130xl*
130 *Genetic Analyzer* (Applied Biosystems) using *genescan 500 LIZ* size standard (Applied Biosystems).
131 Microsatellite alleles were scored using *genemapper* Software version 4.0 (ABI Prism).

132 **Coefficient of relatedness.** Prior to the relatedness analysis, a simulation was performed to provide an
133 assessment of different estimators of relatedness coefficients (r_{xy}). This standard approach by
134 simulation determined the most appropriate estimator for our dataset. Given the allelic frequencies
135 within the population (from the sample sizes $n_{SP1-5} = 264$ and $n_{SP1-6} = 262$), 2,000 individual genotypes
136 were simulated. From the simulated genotypes, 1000 pairs (or comparisons between two simulated
137 individuals) were drawn for four relationship categories (unrelated, half-siblings, full-siblings and
138 parent-offspring) and r_{xy} was calculated for each pair within each relationship category. The

139 calculation of r_{xy} for each pair, within the four relationship categories listed above, was performed
140 using six separate estimators (Lynch 1988; Queller and Goodnight 1989; Li et al. 1993; Ritland 1996;
141 Lynch and Ritland 1999; Wang 2002; Milligan 2003) as described in (Wang 2011). All simulations
142 and calculations of r_{xy} for the empirical dataset were conducted using the package *related v0.8* (Pew et
143 al. 2015), the implementation of the software Coancestry (Wang 2011) in R. The Triadic Likelihood
144 method - TrioML was the most appropriate at describing the known relatedness in our simulated data
145 and thus used to describe the coefficient of relatedness between males and females in the same mating.
146 Hence, we compared the coefficient of relatedness of individuals in mating to the coefficient of pairs
147 of randomly associated males and females from the same population.

148 ***Paternity assessment.*** Based on the genotypes obtained for the coefficient of relatedness, we selected
149 polyandrous matings that allowed assessing the paternity of each egg in the ephippia from
150 microsatellites. We performed the same genotyping as above, but on the oocyte.

151 ***Statistical analysis.*** All analyses were performed using R and Rstudio (RStudio Team 2016; R Core
152 Team 2019). Supplementary material 1 was generated by Rmarkdown, a dependence of RStudio, and
153 provides a summary data table, all scripts associated to their analyses and plots, including
154 supplementary figures. All the analysis and illustrations were done using the tidyverse R package suite
155 (Wickham 2016; Wickham and Henry 2019; Wickham et al. 2019). We used the *Viridis* color palette
156 to make plots easier to read by those with colorblindness and print well in grey scale (Garnier 2018).
157 To illustrate the difference between factors in paired analysis, we additionally used the estimation
158 graphic methods as in Ho *et al.* (2019) with the package *dabestr v0.2.2*. This method uses bootstrap to
159 estimate the difference between means and its 95 % confidence interval. Although not perfect to
160 illustrate complex mixed models, it helps to represent the effect of paired comparison and the
161 confidence we can have in it. Odds ratios quantify the relation between two factors and typically
162 quantify the effect of a variable.

163 Generalized mixed models were fitted using the function *fitme* from the package *spaMM v2.6.1*
164 (Rousset and Ferdy 2014). This function allowed us whenever necessary to include random effect in
165 mixed model, notably to pair the variable by mating or pool it by population (with the argument “1|”),

166 to nest variables (with the argument “/”), to specify the family of the random effect (with the argument
167 “rand.family”) and to consider heteroscedasticity (with the argument “resid.model”). The significance
168 of the factors in the model was tested using a likelihood ratio test, which compares the model with and
169 without the variable of interest.

170 *Sperm length*. Based on AIC criteria, the sperm length was best fitted with a Gamma distribution. In
171 males of the same mating, we tested if there was a difference between the first and second male to
172 detach using the model: Sperm_length ~ Position_detached + (1|ID_mating),
173 family=Gamma(link="log"), rand.family= gaussian("identity").

174 In males from several lineages raised in laboratory conditions (AM-AR initially sampled from
175 Armenia, CY-PA-1 from Cyprus, DE-Iinb1 from Germany and RU-KOR-1 from Russia), the model
176 included the differences in variances among clones as follows: Sperm_length ~ Clone + (1|Clone/ID),
177 resid.model= ~ Clone, family= Gamma(link= "log"), rand.family= Gamma(link= "log").

178 Sperm length varied considerably within an ejaculate. To investigate this variation within each
179 ejaculate, we fitted a gamma or a normal distribution on sperm length data for each individual male
180 using the function *fitdistr* from the package *fitdistrplus v1.0.14* (Delignette-Muller and Dutang 2015).
181 We then tested the goodness of fit of this distribution with the function *gofstat* from the same package.
182 The final AIC for each distribution was obtained by summing the AICs obtained for distinct males. To
183 test if there were potentially two sub-populations of sperms inside one ejaculate, we compared the AIC
184 of the best model to that of a mixed model considering two gaussian distributions. The fit was
185 performed with the function *densityMclust* from the package *mclust v5.4.5* (Scrucca et al. 2016) and
186 the AIC was calculated.

187 *Body length*. The body length of mating males was best fitted with a Gaussian distribution and by
188 considering the difference in variance among populations. The full model to test if there was a
189 difference between males in the same mating was as follows: Body_length ~ as.factor(Nbr_of_males)
190 + (1|Population), resid.model= ~ Population, family= gaussian(link=identity), rand.family=
191 gaussian(link=identity).

192 The relation between spine and body length of males and females was best fitted with a Gaussian
193 distribution and by considering the difference in variance among populations. The full model to test if
194 there was a difference between mating individuals and those single in the population was as follows:
195 $\text{Spine_length} \sim \text{Body_length} + \text{Sex} + \text{Mating_status} + (1|\text{Population})$, $\text{resid.model} = \sim \text{Population}$,
196 $\text{family} = \text{gaussian}(\text{link} = \text{identity})$. The significance of the factors in the model was tested using a
197 likelihood ratio test, which compares the model with and without “Mating status” as a variable.

198 *Infectious status*. The sex-ratio of the 27 populations in relation to the prevalence of *H. tvaerminensis*
199 in females single in their population was best fitted with a Gaussian distribution. The full model to test
200 if there was a correlation with the prevalence in single females was as follows: $\text{Sex_ratio} \sim$
201 $\text{Population_size} + \text{Prevalence_Female}$, $\text{family} = \text{gaussian}(\text{link} = \text{identity})$. The significance of the factors
202 in the model was tested using a likelihood ratio test, which compares the model with and without
203 “Prevalence_Female” as a variable.

204 The prevalence in males and females single or during the mating process was best fitted with a
205 binomial distribution, noting the presence/absence (1 vs 0) of each individual. “Population” was
206 considered as a random effect in order to pair the analysis. The full model to test if prevalence was
207 different between sexes was as follow: $\text{Infectious_status} \sim \text{Sex} + (1|\text{Population})$,
208 $\text{family} = \text{binomial}(\text{link} = \text{"logit"})$, either only with single individuals or only with mating individuals.

209 We used the same approach to test for a difference in prevalence between mating and non mating
210 individuals, the full model being then: $\text{Infectious_status} \sim \text{Mating_status} + (1|\text{Population})$,
211 $\text{family} = \text{binomial}(\text{link} = \text{"logit"})$, $\text{rand.family} = \text{gaussian}(\text{link} = \text{"identity"})$, with both sexes analyzed
212 separately.

213 Assortative mating regarding the infection status was tested by evaluating the prevalence in males
214 during mating when attached to an infected vs an uninfected female. Population was considered as a
215 random effect to take into account the differences in prevalence among populations and the ID of the
216 mating pair was nested in the population in order to pair the analysis. The full model to test for
217 assortative mating was as follows: $\text{cbind}(\text{Nbr_inf_M}, \text{Nbr_uninf_M}) \sim \text{Infection_Female} +$

218 (1|Pop/ID_mating), family= binomial), rand.family= gaussian (link= identity), where
219 “cbind(Nbr_inf_M,Nbr_uninf_M)” is a way to take into account the number of infected males
220 considering the total number of males in the mating.

221 Results

222 Mating formation

223 Most of the here reported results were obtained from studies in natural rockpool populations of
224 *Daphnia magna*. At the time we sampled (Summer), the average proportion of males was around 30
225 %, ranging from 5 to 60 % across populations (Figure 1A). The shallow rockpools of this
226 metapopulation allowed us to catch pairs of mating *Daphnia* and observe in glass vials the separation
227 of the pair, the egg laying and the release of the resting egg cases. Most matings involved one male
228 (i.e. monandrous mating), but mating with two males were frequent (i.e. polyandrous mating, Figure
229 1B). In rare cases, we found three males in the same mating (seven times out of the 968 matings in the
230 study). Our sampling design did not allow us to estimate the frequency of polyandrous mating, thus,
231 we could not determine which parameters influenced it.

232 We found that in 80 % of the cases (382/477) mating females showed the typical morphological
233 changes of the brood pouch associated with the formation of a resting egg case (ephippium),
234 suggesting that they were ready to mate. Since typically less than 10 % of the adult females in a
235 population are in this stage, this finding indicates that mating pairs do not form randomly. Contrary to
236 males of the cladoceran *Moina brachiate*, which are suspected to detect the reproductive status of the
237 females (Forró 1997), males of *D. magna* seem to search for mates randomly. It is therefore likely
238 that, in *D. magna* as in *D. pulicaria* (Brewer 1998), it is a female's choice to accept a mating attempt
239 or to escape it and that they would accept more likely if they are in the right stage of the sexual
240 process. The other 20 % of matings were with non-reproductive females (23/477) or with females
241 reproducing asexually (73/477). If females try to escape males in case they are not in the right stage,
242 then these matings may represent cases where males enforced matings, but did not realize that this

243 cannot lead to any fertilization success. Out of the 17 matings with females in asexual reproductive
244 mode from which we recorded the time before male departure, 13 lasted more than 10 minutes,
245 suggesting that males did not realize that the females were not in the appropriate stage.

246 **Role of body and spina length.** To test if formation of mating pair is mediated by body
247 length, we assessed the departure of body length relative to the average body length of males and
248 females randomly caught in the population (Figure 2A, supplementary material 1 – section 1.3.2).
249 Mating females were on average 9.5 % larger than those randomly caught in the population and
250 mating males were on average 2.3 % larger than those randomly caught in the population. For females,
251 this means that older females produce resting eggs. For males, this suggests that larger males are more
252 successful in attempting to mating. The same analysis revealed assortative mating regarding body
253 length: larger than average males pair with larger than average females. The strength of this
254 homogamy (15 %, as described by the estimate of the Pearson correlation) is lower than the average
255 strength regarding size-related homogamy across animal taxa (31 % according to Jiang *et al.* (2013))
256 and depends on the population (See supplementary material 1 – section 1.3.3). As larger males may
257 have better access to females, the strength of the homogamy could be lowered by large males also
258 potentially catching small females. Controlling for the average body length of mating males in each
259 population, we tested whether females mating with two males carried smaller males than those
260 carrying one male. Males in polyandrous mating were 10 μm smaller on average than males in
261 monandrous mating, a tiny and not significant difference (Figure 2B). However, polyandrous matings
262 include males that were first alone on the female and expected to be of approximately the same length
263 as males in monandrous matings. We thus tested and found that males from the same mating were
264 different in body length (Figure 2C). The males departing second are on average 1.3 % larger than the
265 first males to detach, suggesting that a larger body length could help remaining longer on the female
266 and potentially give advantage in competition for egg fertilization. We further tested whether the
267 length of the tail spine (spina) could be a trait affecting the access to females. We tested whether
268 individuals with longer spine were more often found mating. To do so, we used relative spine length
269 and subtracted the mean value of individuals of the same sex caught randomly in the population. We

270 found that relative spine length was generally shorter for mating individuals (Supplementary material
271 1 – section 1.3.6). Altogether, these results suggest that body length plays a role in *D. magna* sexual
272 selection in the rockpool metapopulation.

273 **Inbreeding avoidance.** The fact that females can potentially choose a particular male opens the
274 possibility for avoiding inbreeding (Duthie and Reid 2016). Inbreeding depression and heterosis have
275 been documented in *Daphnia magna* and avoiding mating with relatives is expected to provide a
276 selective advantage (De Meester 1993; Ebert et al. 2002; Haag et al. 2002). We investigated if this
277 occurs in two natural populations by sequencing four polymorphic microsatellites loci (see details in
278 tables in supplementary material 1 – section 4) and testing if females were mating with males less
279 related than expected by chance. The individuals were either caught in the process of mating (Pop
280 SP1-5: 85 females – 147 males; Pop SP1-6: 92 females – 138 males) or single (16 males for each
281 population). Our result suggests that individuals forming naturally mating pairs were not less related
282 than random mating simulated *in silico* (Wilcoxon test: Pop SP1-5: $W=17085$, $p=0.6$; Pop SP1-6;
283 $W=16814$, $p=0.17$. See supplementary material 1 – section 1.4).

284 **Parasite infections.** We found that the prevalence of *Hamiltosporidium tvarminnensis*, a common
285 microsporidian infection in this metapopulation, was on average around 40 %, ranging from 0 to 100
286 % of individuals, with both sexes being infected (Supplementary material 1 – section 1.5.1). Thus, *H.*
287 *tvarminnensis*, a parasite mediating selection in the here studied metapopulation (Cabalzar et al. 2019),
288 was frequent at the moment of our study, in agreement with (Ebert et al. 2001; Lass and Ebert 2006).
289 Roth *et al.* (2008) showed in laboratory experiments that infected females produce more sons. This
290 implies indirectly that the prevalence of the parasite in a population should correlate positively with
291 the number of males relative the number of females. Contrary to this expectation, we found that the
292 prevalence of *H. tvarminnensis* in single females does not correlate with the sex-ratio in the population
293 (Figure 3A) This suggests that even if individual infected females produced more infected sons, the
294 production of males was compensated at the population level, in accordance with Booksmythe *et al.*
295 (2018) showing that population of *D. magna* are able to adjust the production of males depending on
296 the current sex-ratio.

297 Prevalence differed between sexes, but depended on population and mating status (binomial
298 glm, interaction Sex x Mating status: χ^2 LRT = 17.9, df=1, p= 0.00002). On average, the prevalence
299 was lower in males than in females in the population (Figure 3B left panel, odds ratio: 0.64), as
300 suggested in (Roth et al. 2008), but this was reversed in mating pairs (Figure 3B right panel, odds
301 ratio: 1.41), albeit this result depended strongly on the population.

302 Parasitism is thought to be a major factor in sexual selection. Either because of its direct cost
303 (i.e. females want to avoid becoming infected) or its direct benefits (i.e. healthy males in an infected
304 population might carry good genes). Males in the process of mating were more often infected than
305 those which were single in the population (Figure 3C left panel, odds ratio: 1.85). Females in the
306 process of mating were about as often infected as those single in the population (Figure 3C right panel,
307 odds ratio= 0.89). This could suggest that infected males are chosen by females as males affording to
308 be infected while attempting to mate with a female maybe particularly strong. There was assortative
309 mating based on the infection status. Taking the infection rate of the population and the size of our
310 samples into account, infected males were significantly more likely to mate with infected females than
311 with uninfected ones (Figure 3D, odds ratio: 1.81).

312 Mating behavior

313 After we lifted matings females from the pond to glass jars, we observed them and recorded the time
314 between the moment we caught them and the moments each male detached (Figure 4). The mean time
315 to detachment was 24 min (\pm 1.9 se), suggesting that the total mating time can be estimated to be twice
316 as long, i.e. about 50 min. This estimate is based on the assumption that males and females in the
317 process of mating were caught randomly in the population and that the time of mating is normally
318 distributed. This corroborates with the range of time before detachment in our dataset which was
319 between few and 60 minutes (excluding a unique outlier of 242 min). Polyandrous matings lasted
320 longer than monandrous matings due to the second male remaining attached for longer, but the first
321 male to detach did it as fast as single mating males. Following detachment of males, females readily
322 laid their eggs into the brood pouch (96 % of the cases) (Figure 5A), with 86 % (45/52) of the females

323 doing so within 10 minutes (Figure 5B). Out of 107 egg cases produced, 93 % contained two eggs, 5
324 % had one egg and 3 % were empty (Figure 5C).

325 Sperm morphology

326 **Fatherhood analysis.** The simultaneous presence of two or more males mounted on the same female
327 is not only a strong indicator for direct male-male competition, but also indicates sperm competition.
328 Consistent with this, we found that in eight egg cases (ephippia), from which we genotyped the
329 mother, the two males, and the two embryos, six were fertilized by only one of the two males attached
330 on the female (full-sibs), the other two embryo pairs were half-sibs.

331 **Sperm length.** We next investigated whether sperm length could be a trait involved in male-male
332 competition (Godwin et al. 2017). Differences in sperm morphology and/or quality between males of
333 the same mating would be the substrate for selection upon sperm competition. Using 46 polyandrous
334 matings from natural populations, we found that sperm length of males from the same mating can
335 differ (Figure 6A). Although it is difficult to tell whether this difference is sufficient, more than 50 %
336 of our couples had a mean difference larger than 0.77 μm (i.e. 8.6 % larger than the averaged sperm
337 length). The average sperm length of the second male to detach was 0.96 times the average of the first,
338 a difference which was not statistically significant ($df= 1$, $\text{Chi}^2 \text{LRT}= 2.9$, $p= 0. 086$).

339 To investigate if there could be genetic variation for sperm length, we investigated the
340 ejaculates of four laboratory-raised clones of *D. magna*. We found that there was more variation in
341 sperm length between clones than between individuals from a same genetic background and that some
342 clones had a higher mean sperm length than others (Figure 6B). Considering single ejaculates, the
343 variation in sperm length is strikingly large (Figure 6C inlet). Sperm length within ejaculates of males
344 from the wild is ranging from 3 to 20 μm with an average of 9 μm (Figure 6C). The averaged standard
345 deviation in sperm length for an ejaculate (i.e. 1.9 μm) was close to the standard deviation calculated
346 across ejaculates of males from three different populations (i.e. 2.2 μm). As large variation in sperm
347 length is often attributed to different sperm subpopulations, we looked at the distribution in sperm
348 length within each ejaculate (Figure 6D only illustrates the distribution of the pooled sperm length).

349 We found that sperm length within ejaculates is better described by a Gamma distribution (Combined
350 AIC per model for each ejaculate was 12096.67) than a Gaussian distribution (Combined AIC per
351 model for each ejaculate was 12218.56) or a mixture of two Gaussian distributions (Combined AIC
352 per model for each ejaculate was 12613.04). Thus, it is less parsimonious to suggest that ejaculates are
353 composed of a mixture of two morphologies, each with different roles in sperm competition.

354 Discussion

355 While studying the biology of cyclical parthenogenetic species, it is often easier to focus on
356 the asexual part of the life cycle. Consequently, the evolution of these species is generally investigated
357 through studies of survival and reproduction (natural selection), without considering the possible role
358 of sexual selection. Working with *D. magna* populations inhabiting shallow rockpools, where sexual
359 reproduction is rather frequent, allowed us to focus on the biology of sexual reproduction of this
360 crustacean in a natural setting and to gain an understanding of the role of sexual selection. Here we
361 present the process of sexual reproduction in a stepwise order and point out the possible mechanisms
362 at work in each step. We summarize the entire process in table 1. We conclude that there is clear
363 evidence that sexual selection plays a role in cyclical parthenogenetic species.

364 **Mating formation.** As is the case in other cyclical parthenogenetic species (Dixon 1977; Ward et al.
365 1984; Snell and Hoff 1985; Hand 1991), environmental conditions trigger female *Daphnia* to switch
366 from producing asexual daughters to producing asexual sons (Ebert 2005). Other females switch from
367 producing asexual daughters to producing sexual eggs that need fertilization by males. Males search
368 for appropriate females by fast swimming. It seems that male-female encounters are random.
369 However, some form of sorting must take place, as mating males are mainly found on females in the
370 late phase of the sexual process, when the structure of the egg case is already visible and oocyte
371 release is imminent. Furthermore, we find evidence for assortative mating for body length and
372 infection status. This suggests that after an initial, presumably random, encounter, males either leave
373 females that do not fit their expectation, or females reject males when not at the right stage or that do
374 not fit their quality assessment. Males are generally not a limiting resource for females, while access to

375 females ready to mate is likely to be limiting for males. Typically, especially when males are
376 numerous, there are more males than females ready to mate in a population at a given moment in time.
377 Hence, whereas males are likely to accept any female in the right stage they encounter, females are
378 likely to be choosy. Consistent with this, during our observations of the rockpool populations we
379 frequently observed females violently shaking off males that attempt to mount them (D. Duneau, D.
380 Ebert, personal observation).

381 Our results show that males in mating pairs are more often infected than single males in the
382 population. This is an unexpected result, as infected males may be weaker and were shown to have
383 reduced sperm counts (Roth et al. 2008). It could suggest that females choose infected males, because
384 infected individuals that are still able to catch a female are likely to be strong males. In this case
385 infection would be an honest signal, a handicap (Zahavi 1975). Alternatively, males may counteract
386 attempts of the female to push them away, and stronger males may also be more able to resist the
387 females attempts to reject them, consistent with our finding that males in the process of mating are on
388 average larger than single males. Thus, it is also possible that infected males still alive and able to
389 catch females are also the strongest males. The parasite cannot spread to the female or her offspring
390 during mating, thus there is no risk associated with mating. In summary, in this phase of the sexual
391 process sexual selection, notably for body length, may be driven by female choice.

392 **Copulation.** With increasing density and proportion of sexual animals in a population, the number of
393 cases with multiple encounters will increase and polyandrous matings become more common. We
394 observed many cases of polyandrous matings involving two males hooked to one female, and some
395 cases with even three or four males. Such polyandrous matings open the door for male-male
396 competition in two forms. First, males may compete directly with each other for the best position and
397 the longest stay-time on a female. We found that the male staying longer on a female is on average
398 slightly larger than the male leaving earlier, which is consistent with the idea that stronger males have
399 more control over the situation.

400 The second form of male-male competition is by sperm competition. If multiple males deposit
401 their ejaculates around the same time into the brood chamber of the female, competition among sperm

402 may favor males with more and/or better sperm. Indeed, we found that in 6 out of 8 cases studied in
403 detail, one male fertilized both eggs. As sperm of *D. magna* seem non-motile, competition is unlikely
404 related to the swimming speed in the brood pouch.

405 **Fertilization.** Our observation regarding fertilization suggest the following sequence. Within ten
406 minutes after a male left, most females release one or two eggs into her brood pouch, which by this
407 time had turned into the future egg case, specialized as a resting structure, the ephippium. The
408 oviducts open into this brood pouch at the caudal end of the structure, close to the place the male
409 attaches to. Fertilization takes place either in the brood pouch (external fertilization) or in the two
410 oviducts (internal fertilization). Fertilization has never been studied in *D. magna*, but for *D. pulex* it
411 was suggested that it occurred internally, before the deposition in the brood pouch (Ojima 1958;
412 Hobæk and Larsson 1990). However, here we argue that fertilization in *D. magna* is likely to be
413 external, although future studies are needed to fully test this hypothesis.

414 We believe that the most parsimonious mechanism for fertilization is that the male ejaculates a
415 large number of sperms into the brood pouch and that upon arrival the oocytes come in contact with
416 the sperm. Several arguments are in support of the brood pouch fertilization hypothesis and the
417 rejection of the internal fertilization hypothesis. First, access to the oviducts with the genital papilla on
418 the male's abdomen is rather limited. The male genital papilla is large and conical shaped and thus
419 would not be able to insert itself into the oviduct which is closed until the eggs is laid (Lee et al.
420 2019). Second, each sexual egg is released from one oviduct and the males could probably only access
421 to the one closer to the side it is attached to. This would strongly reduce the possibility for having both
422 eggs fertilized in case only one male is attached. However, as typically both eggs can hatch, they must
423 both be fertilized. Also, our finding that in double matings the two eggs are often fertilized by the
424 same male, would not work with the need for the male to insert its papilla into the ovary. Third, the
425 sperm is apparently not motile. It is therefore unclear how the sperm could find the oviduct and travel
426 within it. Fourth, the shape of the sperm is not streamlined to move in one direction. It is oval to short
427 rod shaped with two blunt ends (Figure 6D). Taken together, we believe that fertilization inside the

428 oviduct is unlikely in *D. magna* and that more likely alternative is that males release their sperm into
429 the brood pouch where they wait for the unfertilized eggs to arrive.

430 How does the sperm meet the oocyte? The brood pouch is part of the outside environment. It
431 is open to the outside and water can freely circulate through it. Sperm may cover the inner lining of the
432 brood pouch, so eggs would touch the sperm as soon as they are released from the ovary. Sperm may
433 also preferentially attach to the area around the opening of the oviduct and fertilize in the moment of
434 egg release. However, there is no obvious structure supporting this speculation. If this would be the
435 case, the first male to attach would have an advantage and mating may not need to last for 50 min on
436 average. Another option is that sperm do not attach to any tissue before the eggs arrive but stay in
437 suspension in the brood pouch and risk that the stream of water that oxygenates the brood pouch
438 flushes them out. For fertilization to occur, the female may either stop this water flow until the eggs
439 are laid and avoid washing all the sperm out or flushing out may select for males producing large
440 ejaculates with high quality sperm. Either way, sperm competition could take place in this selection
441 arena, with ejaculate quality, sperm quantity and time of ejaculate deposition being crucial aspects for
442 fertilization success.

443 Sex-ratio can be highly variable among *D. magna* populations and it is also known to vary
444 strongly over the season (Booksmythe et al. 2018). Consequently, the intensity of sperm competition
445 may vary among populations and over time. When males are numerous, they must compete to fertilize
446 the oocytes in polyandrous matings. Those with the highest number of sperms remaining in the brood
447 pouch when the female lays the eggs, after their departure, are likely to have the highest fertilization
448 success. This would favor males producing more sperm and males staying longer on the females.
449 When those polyandrous matings are less common, sperm released in the female brood pouch can still
450 be flushed out, it is then likely that the ejaculate evolved a higher sperm number, but because of sperm
451 limitation (as defined in Liao *et al.*, 2018), not due to sperm competition. However, the difference in
452 optima for sperm number upon selection by sperm limitation or by sperm competition is unknown.

453 As most sperm production is done early in a male's life (Wuerz et al. 2017) the total number
454 of sperm is limited by the size of the spermiduct. In *Daphnia*, there is an extracellular compaction

455 process by a vacuole before the mature sperm is released into the spermiduct which maximizes the
456 amount of stored sperm (Wingstrand 1978). Even though the sperm that we observed in *Daphnia*
457 medium remain intact, it is expected that the vacuole opens in the brood pouch, eventually upon
458 contact with the oocyte. Hence, sperm number is tightly correlated with sperm length, or at least to its
459 compaction. When sperm number is under strong selection, directional selection is expected to select
460 for an optimal length considering the optimal sperm number. However, if precisely controlling sperm
461 length is costly, the amount of variation in sperm length is predicted to correlate negatively with the
462 intensity of sperm competition (Bauer and Breed 2006; Fitzpatrick and Baer 2011; Varea-Sanchez et
463 al. 2014; Rowley et al. 2019). The high variance in sperm length we observe here could therefore
464 indicate weak sperm competition overall. In such a case, this rod-like cell does not have to have a
465 strict morphology and consequently will have a more or less condensed sperm shape.

466 **Sexual selection gradient in each sex.** A. J. Bateman articulated several principles to explain when
467 males have an indiscriminating ardor to obtain mates, while females are expected to be choosy
468 (Bateman 1948). In *Daphnia*, the relationship between mating success and reproductive success in
469 females, is what he called the single-mate saturation (Bateman 1948). In this relationship, a single
470 mating is enough to fertilize the entire clutch and there is no increase in reproductive success once the
471 individual female obtained one mating partner. Whether or not females benefit from male-male
472 competition by having additional males in the same mating is not clear. Their clutch size is unaffected;
473 only the paternity is potentially divided between the inseminating males. The sexual selection gradient
474 in female *Daphnia* should therefore be weak (See figure 2 in Arnold, 1994). In males however, the
475 expected reproductive success will increase linearly with the number of matings in the absence of
476 sperm competition. When polyandrous matings are common, the relationship between mating success
477 and reproductive success in males is what Bateman called the diminishing returns (Bateman 1948). In
478 that case, as ejaculate size diminishes with the number of matings the competitiveness of the males
479 mechanically decreases as well as the potential number of offspring gained with each additional mate
480 (Arnold 1994). Hence, male mating success will correlate linearly with reproductive success when

481 males are proportionally less numerous than females ready to mate, while the relationship will saturate
482 in presence of male-male competition.

483 **Sexual selection in other cyclical parthenogenetic species.**

484 Only little is known about sexual selection in other cyclical parthenogenetic species but there are some
485 reasons to believe that it plays an as important role as in *Daphnia*. Aphids are probably the cyclical
486 parthenogenetic species about which we know the most. First, some species of Aphids can recognize
487 specific mates (Guldemon et al. 1994). Also, females have been shown to release species-specific sex
488 pheromones daily and at specific time to call males (Guldemon and Dixon 1994). Those two
489 mechanisms used to reduce interspecific insemination may had a role in speciation by reinforcement in
490 aphids (Guldemon and Dixon 1994). They could also be used by females to choose mates within the
491 same species. It is known that Aphids can mate for more than ones and display some form of pre-
492 copulatory stroking behavior but the occurrence of sperm competition seems unknown (Doherty and
493 Hales 2002). Female choice is certainly possible as females can avoid inbreeding and refuse to mate
494 with certain males (Huang and Caillaud 2012). The example of *Daphnia* and the few elements in
495 Aphids suggest that sexual selection has probably a role in the evolution of cyclical parthenogenetic
496 species. Considering that sexual reproduction is generally associated with the capacity to respond to
497 environmental changes or with dispersal, this role may be more important than we thought until now.

498 **Conclusion**

499 Cyclical parthenogenesis, the strategy in which organisms go through several rounds of clonal
500 reproduction before a sexual event, is a widespread form of reproduction in many taxa, including
501 crustaceans, rotifers, aphids, and in human parasitic nematodes. While often neglected, we argue that
502 sexual selection is an important form of selection also in these cases of occasional sexual reproduction.
503 It is possible that the temporary absence of sexual reproduction in cyclical parthenogenes gives a non-
504 negligible role to drift in the evolution of their sexually dimorphic traits. This is because, when the
505 sexual event occurs only after several generations of clonal reproduction the intensity of sexual
506 selection on sexual traits is overall reduced. Reduced intensity of sexual selection may allow the

507 accumulation of genetic variation allowing selection to occur on multiple alleles at ones. This is a
508 principle reminiscent of the two first phases of Wright's shifting-balance theory (Wright 1982).
509 Furthermore, it is likely that during clonal reproduction, sexual traits (e.g. body size) may deviate from
510 their optimal for mating/fertilization success because they have different optimum under natural
511 selection.

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519

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- 719

720 Tables

721 **Table 1:** Description of the sexual process in *Daphnia magna*.

	Process	Open question	
Mating formation	Mate search	Males seem to search randomly for females that are ready to lay sexual eggs (not investigated here).	Do males search randomly for mating partners?
	Mate encounter	In some species, males seem to be able to follow the current generated by the escaping female (not investigated here).	
	Mounting	There is a possibility for female choice here. Males are rarely found attached to asexual females. It seems unlikely that males are choosy as females ready to mate are a limiting resource. Females can escape from males attempting to mount them. Females are found with males larger than the population average and/or with males sharing the same infection status. There is no evidence for a deviation from random mating regarding genetic relatedness.	Do females actively reject males based on quality criteria?
Copulation phase	Copulating	Males remain attached to the female for about 50 min. There is a possibility for male-male competition when several males attach simultaneously to the female. In this case, the male that stays longer on the female is typically larger, which may give it an advantage to fertilize the egg. Sperm competition may start here, but its intensity depends on the frequency of polyandrous matings. In polyandrous matings, one male can fertilize the two eggs.	When does ejaculation happen during the 50 min mating period? What are the male traits favoring fertilization success?
Post copulation phase	Egg laying	By laying their sexual eggs only after the male(s) have left, females may be able to execute cryptic choice. The water flow circulating in the brood pouch (for oxygenation) may select for sperm number and quality. If the high variation in sperm length comes from the absence of a quality control mechanism, then females may select for good sperm by flushing those that are not suitable out (e.g., not able to stick on the cuticula until the egg is laid). The fact that sperms can be flushed out may also select for large ejaculates.	What happens to the sperm before the oocytes arrive in the brood pouch?

It likely occurs in the brood pouch, not in the oviduct, and hence is a form of external fertilization.

**Egg
fertilization**

Does the fusion of the gametes occur at any points of the oocyte or at a specific place? This could illuminate the role of sperm quantity in the ejaculate and the level of stochasticity in sperm competition.

Does sperm length variation correlate with the intensity of sexual selection in the population?

723 **Figure legends**

724 **Figure 1:** Mating in *Daphnia magna*. A/ Proportion of adult males in *Daphnia magna* rockpool
725 populations. Random sampling of rockpool populations showed that the proportion of males was on
726 average about 30 %, ranging from 5 to 60 %. Boxplot divides the dataset into quartiles. It represents
727 the minimum and maximum, as well as the first quartile (25 % of the dataset lies below it), the median
728 and the third quartile (75 % of the dataset lies below it). Each grey dot represents one population and
729 the red dot is the arithmetic mean. B/ Photograph of a female (large individual) mating with two males
730 (small individuals). Females can mate with one (most common), two or even three males.

731

732 **Figure 2:** A/ Role of body length in the sexual process. Mating females were 9.5 % larger than those
733 randomly caught in their population. Mating males were on average 2.3 % larger than those randomly
734 caught in their population. Controlling for average body length in a population, there was positive
735 assortative mating regarding body length: males larger than the average of the population were mating
736 with females larger than the average of the population. B/ Body length and mating types. Males in
737 polyandrous mating were 0.01 mm smaller on average than males in monandrous mating, a difference
738 which was not statistically significant. C/ Difference in body length of the first male to detach minus
739 the size of the second. Males from the same mating are more different than expected by chance (Paired
740 t-test: $df=97$, $t= -0.02$, $p= 0.03$). The second males are on average larger than the first males to detach
741 (mean of the differences= 0.02 mm or 1.3 %) suggesting that body length could remain longer on the
742 female and possibly gain an advantage in competing for egg fertilization. The red line displays zero
743 difference.

744

745 **Figure 3:** Role of the parasite *H. tvarinnensis* in the sexual process. A/ Correlation between the
746 prevalence in single females in the population (i.e., proportion of infected females/total of single
747 females) and the proportion of males in the population ($df= 1$, χ^2 LRT= 0.064, $p= 1$). B/ Sexual
748 dimorphism in infection prevalence in populations of individuals single or mating. Prevalence can be

749 different between sexes, but it depended on the mating status (Sex x Mating status: $df=1$, Chi^2 LRT =
750 17.84, $p= 0.00002$). On average, the prevalence was higher in females than in males in the populations
751 (left panel, $df=1$, Chi^2 LRT = 17.84, $p= 0.00012$, odds ratio: 0.64), but the tendency seemed reversed
752 in mating (right panel, $df=1$, Chi^2 LRT = 4.7, $p= 0.03$, odds ratio: 1.4). C/ Prevalence in single vs
753 mating individuals. On average, the prevalence in males in mating was higher than in single males
754 (left panel, $df=1$, Chi^2 LRT = 17, $p= 4.8e-5$, odds ratio: 1.8). This difference was not found in
755 females (right panel, $df=1$, Chi^2 LRT = 0.49, $p= 0.49$, odds ratio: 0.89). D/ Assortative mating
756 regarding infection. The odds of infected to uninfected males are on average 1.8 higher when the
757 female is infected ($df=1$, Chi^2 LRT = 5.2, $p= 0.023$, odds ratio: 1.8). Histograms underneath graphs
758 are an estimation graphic methods as in Ho *et al.* (2019) using bootstrap to estimate the difference
759 between means and its 95 % confidence interval. We illustrated the population size (N) by the size of
760 the circles, which is taken into account in the statistical analysis. We illustrated the significance of the
761 mixed models when $p\text{-value} >0.05$, with * when <0.5 , and *** when <0.0001 .

762

763 **Figure 4:** Duration of the mating process. In monandrous mating, males detached on average 24 min
764 after they were caught, like the first males in polyandrous mating which detached on average 23 min
765 after they were caught (Wilcoxon test: $W= 3676$, $p= 0.99$). Second males detached on averaged 39
766 min post capture (Wilcoxon test: $W= 4192$, $p= 0.0002$). Those results suggest that mating lasted on
767 average around 50 min. Each dot represents a male in a mating. The y-axis is on a log scale for better
768 illustration.

769

770 **Figure 5:** A/ Number of females laying eggs after the male has left. The large majority of females
771 caught while mating laid their eggs only after the male left. B/Time between the last male detached
772 from the female and the female depositing sexual eggs. 86 % (45/52) did so within 10 minutes after
773 mating (~40 % in less than 5 min). The red line represents the median. C/ Distribution of egg numbers

774 in the ephippium from females caught in the process of mating. Most females laid two sexual eggs.

775 Numbers within brackets represent sample sizes.

776

777 **Figure 6:** Sperm length of *Daphnia magna*. A/ Difference between means ejaculates from males
778 naturally caught in the same mating (first male to detach from the mating minus the second). The
779 difference in sperm length between males in the same mating was larger than 0.77 μm (i.e. 8.6 %
780 larger than the averaged sperm length) in more than 50 % of the cases. However, the position to detach
781 did not predict the direction of the difference as the second male to detach was 0.96 of the average of
782 the first, a difference which was not statistically significant (df= 1, Chi² LRT= 2.9, p= 0. 086). B/
783 Sperm length in ejaculate of males of four clones under laboratory conditions reveals more variation
784 between than within clones (df= 3, Chi² LRT= 17, p= 6e-4), indicating a genetic component of sperm
785 length. C/ Sperm length in ejaculates of males caught in while mating from three natural populations.
786 Sperm lengths were determined after males detached from females using nicotin solution in the
787 laboratory. The males are ranked by median length within their ejaculate. The average standard
788 deviation within an ejaculate was 1.9 μm , about as large as the standard deviation of all the measured
789 sperm, 2.2 μm . D/ Distribution of sperm length of sperm from all males represented in C. The
790 distributions within ejaculates were generally better described by a Gamma than by a Gaussian
791 distribution (or by a mixture of two Gaussian distributions) excluding the hypothesis of two different
792 morphs, with possibly different functions. The inset photograph is an illustration of the sperm length
793 variation of a typical ejaculate.

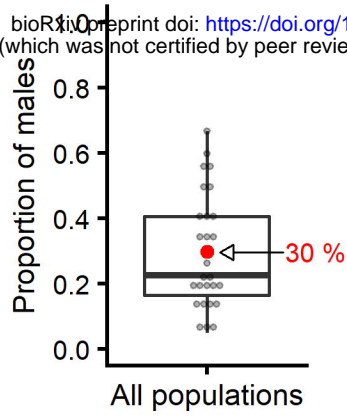
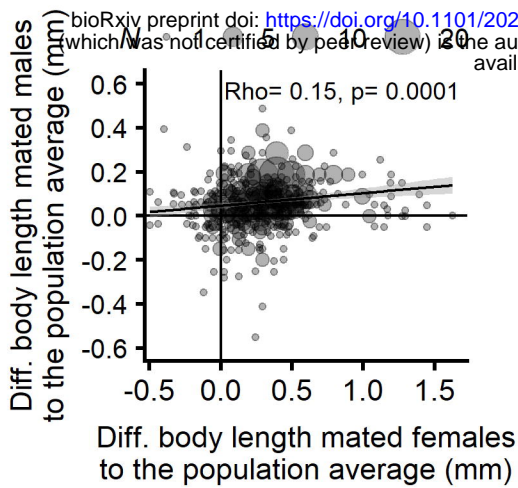
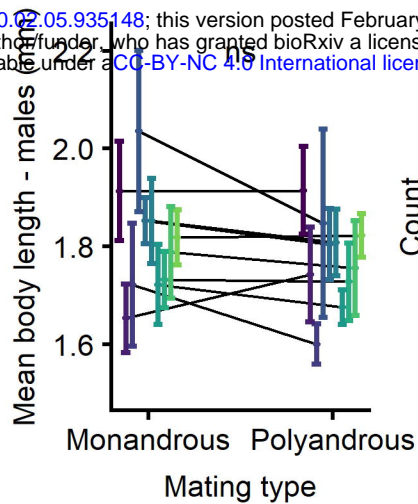
A**B**

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A



B



C

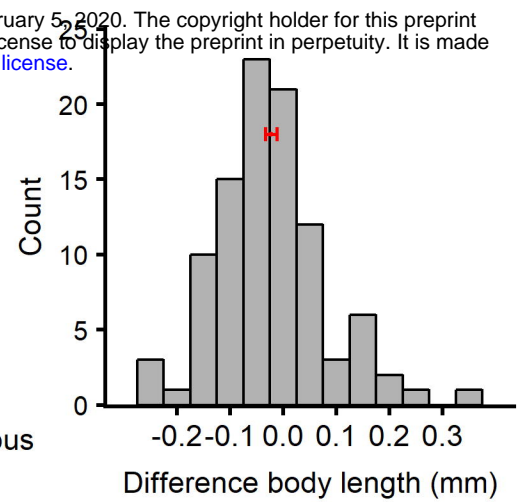
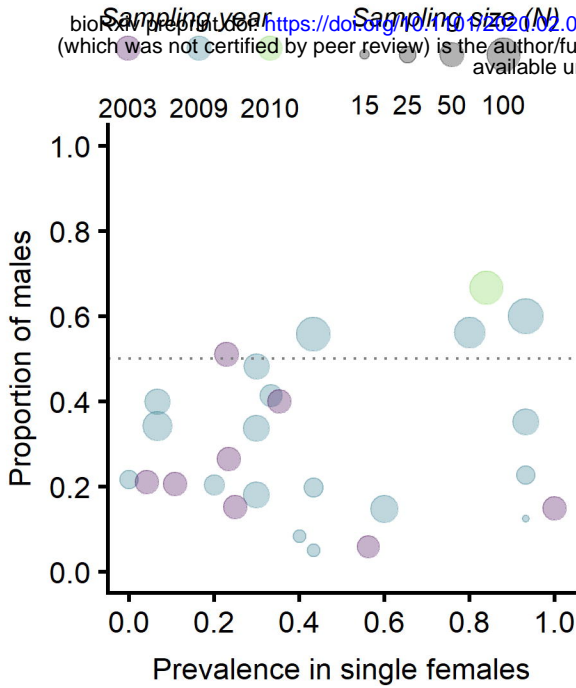
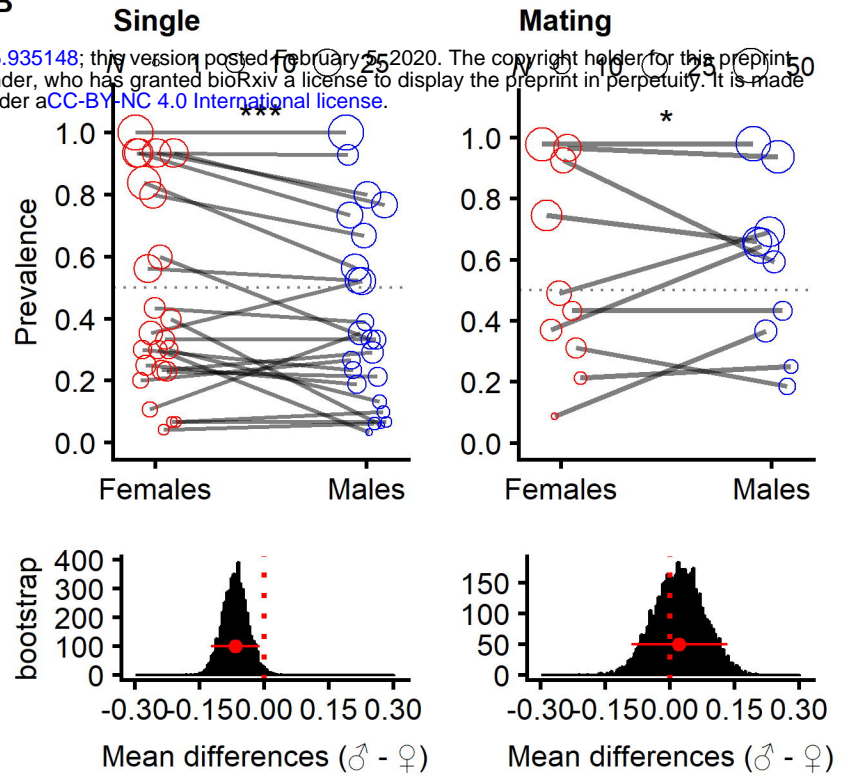


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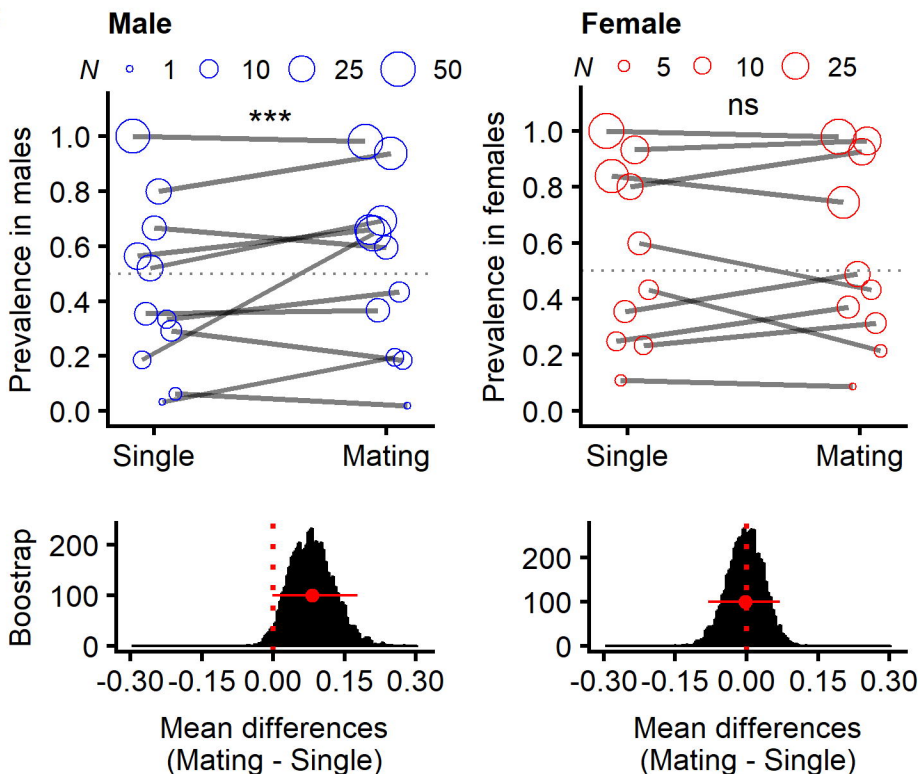
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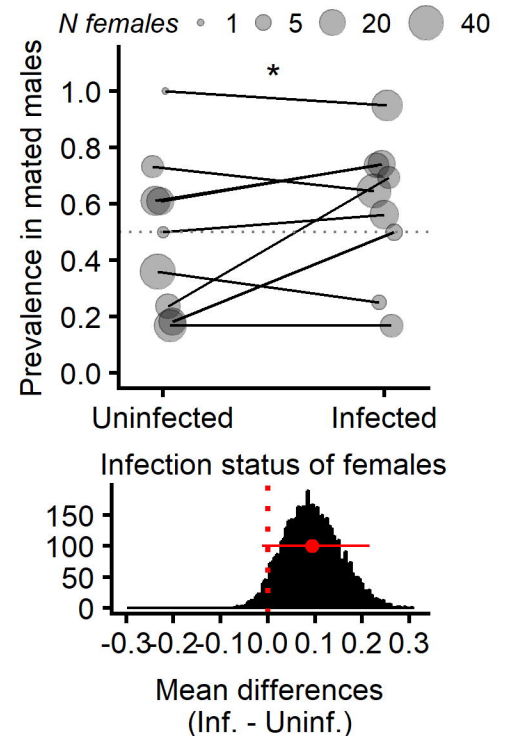


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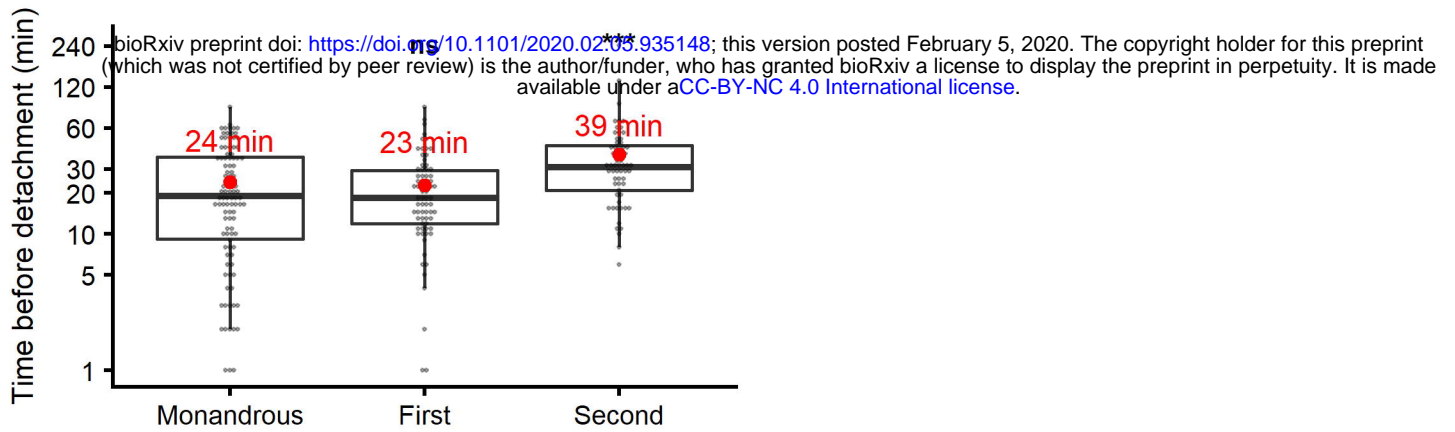


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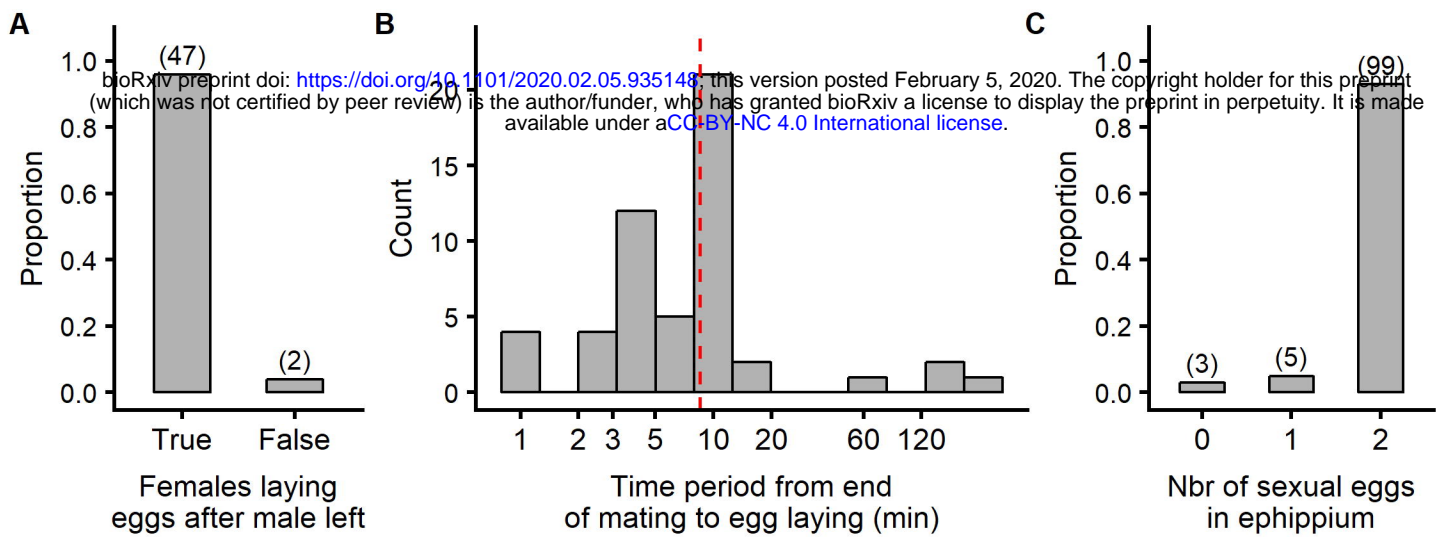


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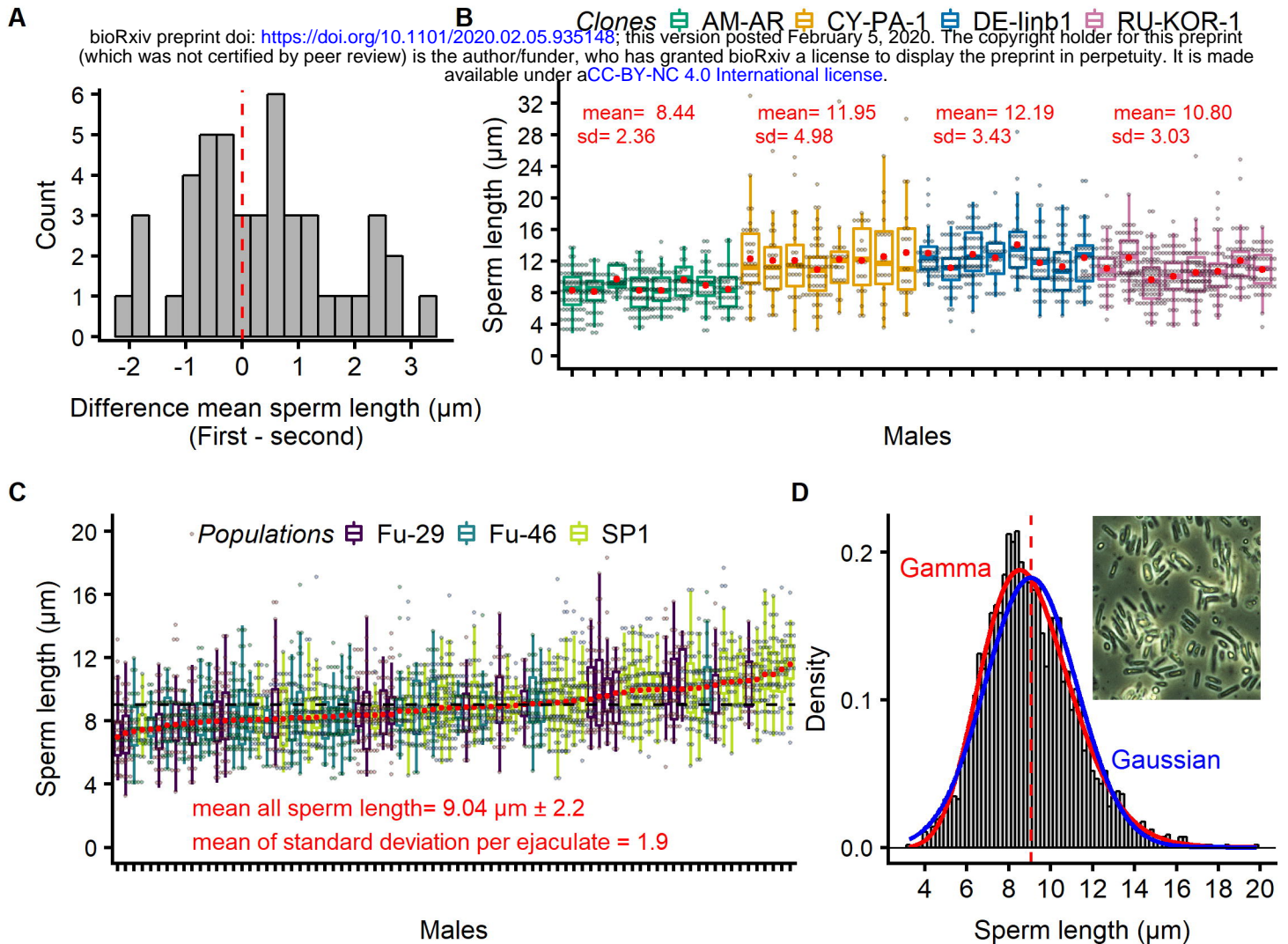


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