Estimation of the propensity for sexual selection in a cyclical parthenogen

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Short title: Sexual selection in *Daphnia*

Abstract

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

Introduction

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

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

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

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

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

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

Materials and methods

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

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

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

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

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

Coefficient of relatedness. Prior to the relatedness analysis, a simulation was performed to provide an assessment of different estimators of relatedness coefficients (r_{xy}). This standard approach by simulation determined the most appropriate estimator for our dataset. Given the allelic frequencies within the population (from the sample sizes $n_{Sp1\text{-}5}$ = 264 and $n_{SP1\text{-}6}$ = 262), 2,000 individual genotypes were simulated. From the simulated genotypes, 1000 pairs (or comparisons between two simulated individuals) were drawn for four relationship categories (unrelated, half-siblings, full-siblings and parent-offspring) and r_{xy} was calculated for each pair within each relationship category. The

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Results

Mating formation

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

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

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

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

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

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

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

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

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

Mating behavior

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

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

Sperm morphology

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

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

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

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

Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Sexual selection in other cyclical parthenogenetic species.

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

Conclusion

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

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

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Tables

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

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Figure legends

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

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

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

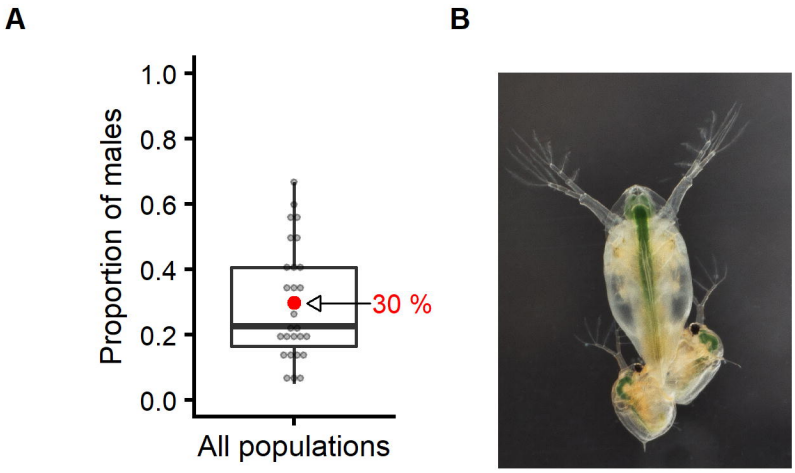
different between sexes, but it depended on the mating status (Sex x Mating status: df=1, Chi^2 LRT = 17.84, p= 0.00002). On average, the prevalence was higher in females than in males in the populations (left panel, df=1, Chi^2 LRT = 17.84, p= 0.00012, odds ratio: 0.64), but the tendency seemed reversed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

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

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

in the ephippium from females caught in the process of mating. Most females laid two sexual eggs. Numbers within brackets represent sample sizes.

Figure 6: Sperm length of *Daphnia magna*. A/ Difference between means ejaculates from males naturally caught in the same mating (first male to detach from the mating minus the second). The difference in sperm length between males in the same mating was larger than 0.77 µm (i.e. 8.6 % larger than the averaged sperm length) in more than 50 % of the cases. However, the position to detach did not predict the direction of the difference as the second male to detach was 0.96 of the average of the first, a difference which was not statistically significant (df= 1, Chi^2 LRT= 2.9, p= 0. 086). B/ Sperm length in ejaculate of males of four clones under laboratory conditions reveals more variation between than within clones (df= 3, Chi^2 LRT= 17, p= 6e-4), indicating a genetic component of sperm length. C/ Sperm length in ejaculates of males caught in while mating from three natural populations. Sperm lengths were determined after males detached from females using nicotin solution in the laboratory. The males are ranked by median length within their ejaculate. The average standard deviation within an ejaculate was 1.9 µm, about as large as the standard deviation of all the measured sperm, 2.2 µm. D/ Distribution of sperm length of sperm from all males represented in C. The distributions within ejaculates were generally better described by a Gamma than by a Gaussian distribution (or by a mixture of two Gaussian distributions) excluding the hypothesis of two different morphs, with possibly different functions. The inlet photograph is an illustration of the sperm length variation of a typical ejaculate.



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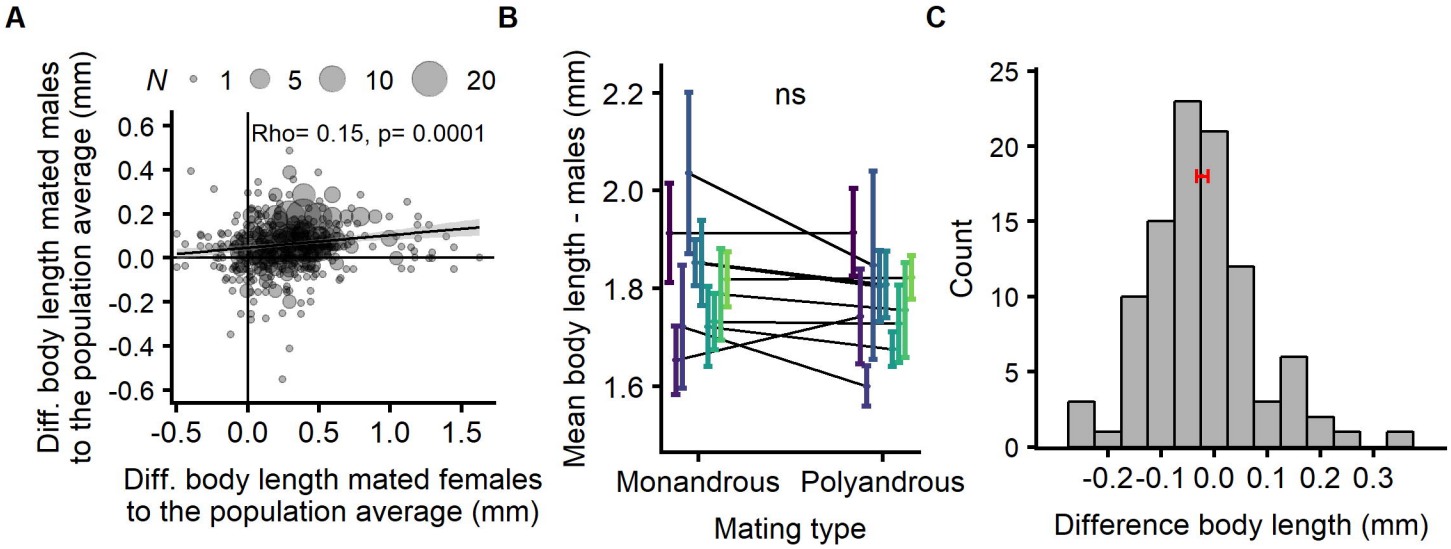

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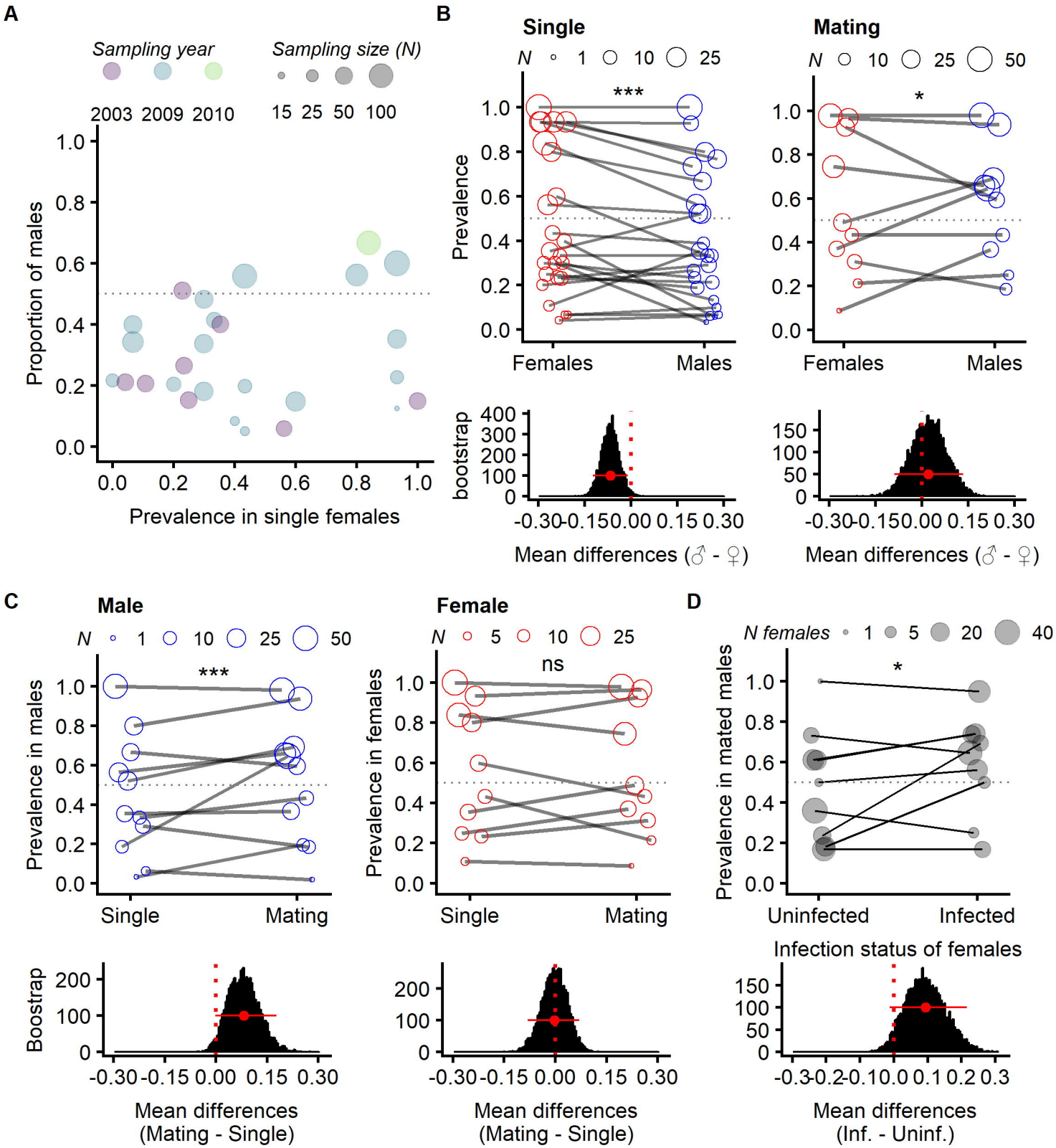

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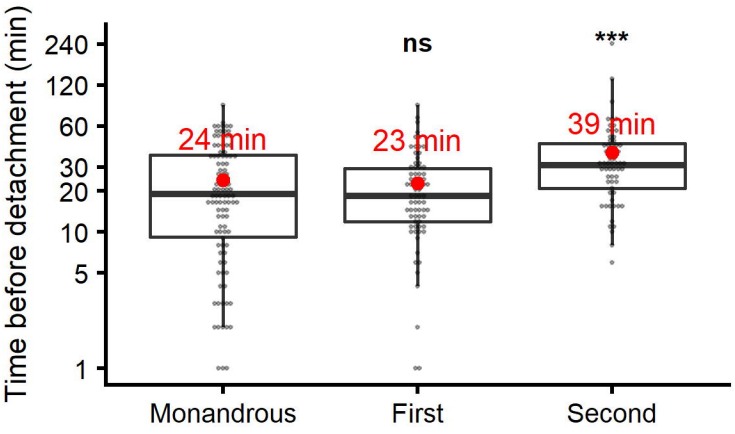

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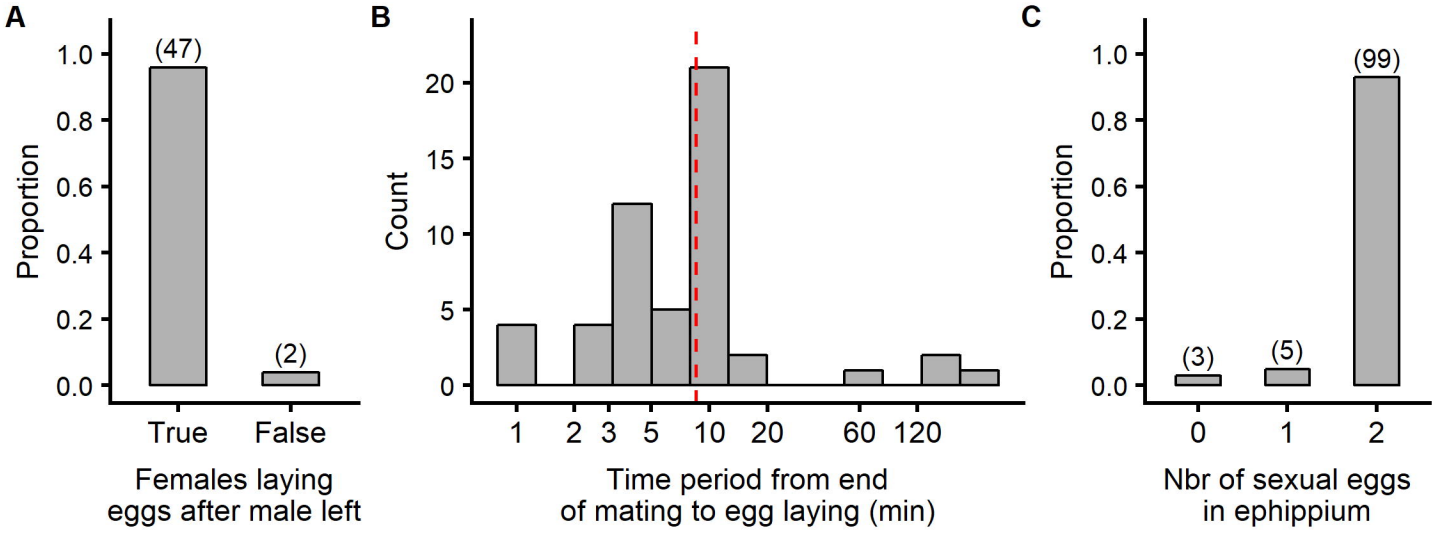

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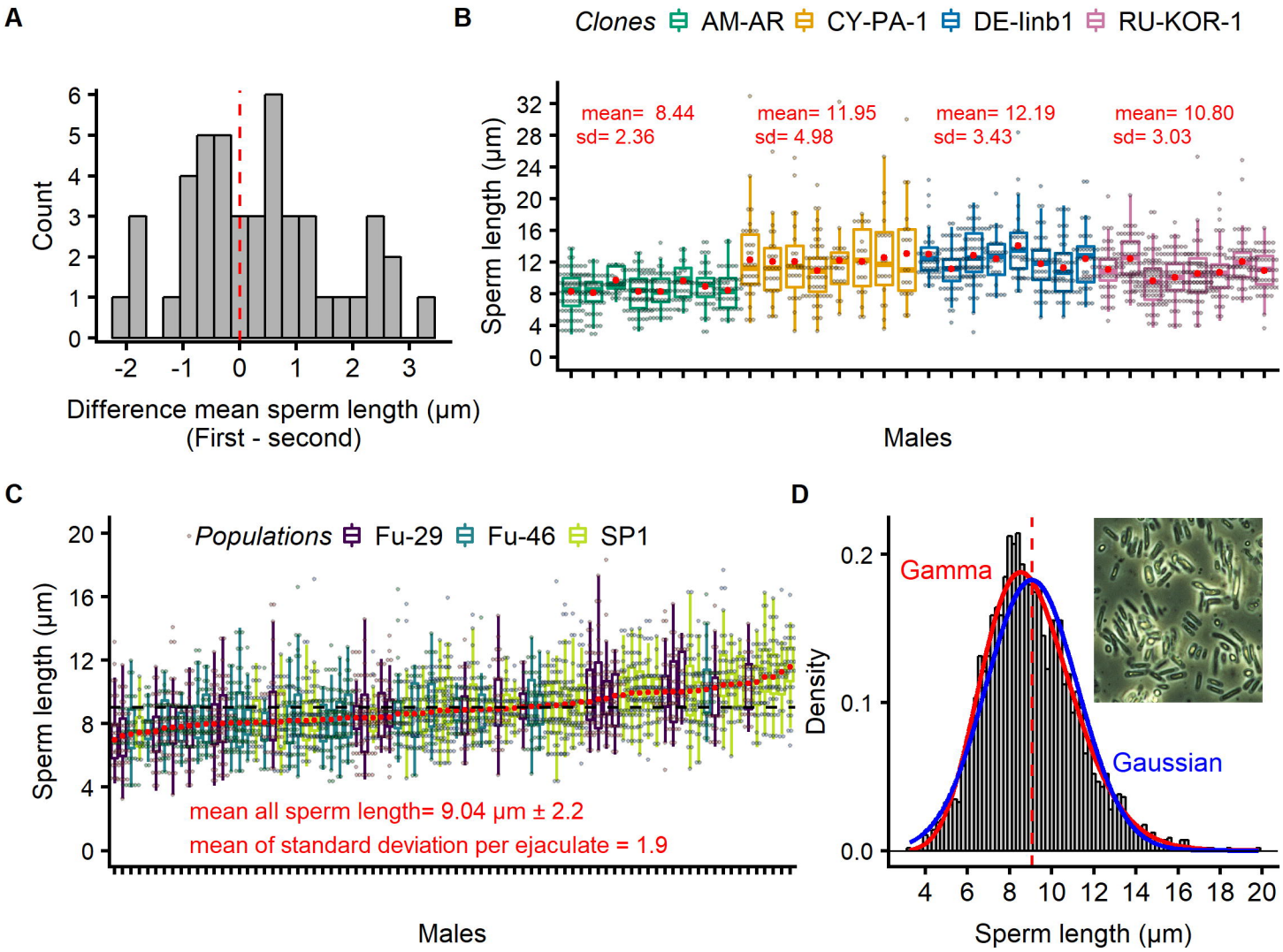

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