When sexual signallers are choosers too

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Mating status affects female choice when females are signallers

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ABSTRACT

Sexual selection in animals has been mostly studied in species in which males are signallers and females are choosers. However, in many species females are (also) signallers. In species with non-signalling females, virgin females are hypothesized to be less choosy than mated females, as virgins must mate to realize fitness and the number of available males is generally limited. Yet, when females signal to attract males, mate limitation can be overcome. We tested how virgin and mated females differ in their signalling behaviour, mating latency, and in mate choice, using the tobacco budworm Chloridea (Heliothis) virescens as an example for a species in which females are not only choosers but also signallers. We found that virgin females signalled longer than mated females, but virgin and mated signalling females were equally ready to mate, in contrast to non-signalling females. However, we found that virgin signalling females showed weaker mate preference than mated females, which can be explained by the fact that females increase their fitness with multiple matings. Mated females may thus further increase their fitness by more stringent mate selection. We conclude that signalling is a crucial aspect to consider when studying female mate choice, because signalling may affect the number of available mates to choose from.

Keywords Chloridea (Heliothis) virescens, female sexual behaviour, female mate choice, mating status, multiple mating, signalling
INTRODUCTION

Female mating decisions are influenced by her mating history (Kelly, 2018; Jennions & Petrie, 1997). When females mate multiple times during their lifetime, their mate choice may depend on whether they are still virgin or have already mated (Jennions & Petrie, 1997), because each mating status has different costs and benefits (Halliday, 1983; Herberstein et al., 2002; Kokko & Mappes 2005; Kelly, 2018). Virgins need to mate to realize fitness (Wickman & Rutowski, 1999) and should thus mate with any male (Jennions & Petrie, 2000; Kokko & Mappes, 2005). Once mated, females can benefit from additional matings (Jennions & Petrie, 2000; Zeh & Zeh, 2001; Parker & Birkhead, 2013; Puurtinen & Fromhage, 2017), because these can provide nutritional or immune boosting nuptial gifts (Brown, 1997; Vahed, 1998; Worthington & Kelly, 2016), compensate for previous, infertile matings (Sheldon, 1994), and provide access to a larger gene pool (genetic benefits, Thornhill, 1983). Multiple matings may also allow females to store sperm from multiple partners and select for paternity (cryptic female choice, Thornhill, 1984; Eberhard, 1996). The genetic make-up of offspring depends on whether the first or the last mated male sires all offspring (first- and last-male sperm precedence), or whether paternity varies (variable sperm precedence) (Parker, 1970; Birkhead & Hunter, 1990; Simmons, 2001). The number of matings and the pattern of sperm precedence can thus affect how much effort a female allocates to precopulatory mate assessment and directs mate choice.

Virgin females and mated females may differ in their level of choosiness, because her sexual behaviour may change once sperm for fertilization is obtained (Jennions & Petrie, 2000; Kokko & Mappes, 2005). For non-signalling females, theory predicts that virgins are less choosy than mated females (Jennions & Petrie, 2000; Kokko & Mappes, 2005). Choosiness can be seen as the effort made by the female to choose a male (Jennions & Petrie, 1997; Widemo & Sæther, 1999; Edward, 2015), which can be reflected in the time until mating (mating latency) (Holveck & Riebel, 2010; Lindström & Lehtonen, 2013), and the strength of mate preference (i.e., the slope of the preference function (Brooks & Endler, 2001; Cotton et al., 2006; Ratterman et al., 2014) or height of the preference function (Kilmer et al., 2017)). For instance, in guppies (Poecilia reticulata) and field crickets (Gryllus bimaculatus), virgin females show weaker mate preference than mated females (Bateman et al., 2001; Pitcher et al., 2003). Also, virgin female red flour beetles (Tribolium castaneum) choose lower-quality males than mated females (Fedina & Lewis, 2008). Such a difference in choosiness between virgin and mated females is expected to be amplified in systems with last-male sperm precedence, because the last male sires all offspring and thus, only the last choice matters (Kokko & Mappes, 2005). A large difference in choosiness between virgin and mated females is indeed seen in female smooth newts (Triturus vulgaris) (Gabor & Halliday, 1997) and fall field crickets Gryllus pennsylvanicus (Judge et al., 2010), which are examples of species with last-male sperm precedence.

The chance of mating may not only increase with a low level of choosiness in virgin females, but also with active signalling by females. Through signalling, females attract males and thus affect male availability. In the extreme case of non-flying females, like glow-worms (South et al., 2011; Hopkins et al., 2015; Baudry et al., 2021; Elgert et al., 2021), praying mantids (Maxwell et al., 2010), or wingless moths (e.g., Roelofs et al., 1982; Wong et al., 1984), signalling is often crucial to secure a mate. The more males a female attracts, the higher her chance for
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mating and the more options to choose a mate. When signalling attracts males of different quality, females may directly compare males and choose for relatively higher-quality partners (reviewed in Jennions & Petrie, 1997). The level of signalling and choosiness are thus crucial elements of female reproductive strategies.

In non-signalling females, female behaviour has no effect on how many males she can choose from, which likely has consequences for her choosiness. Non-signalling females may mate with any arriving male, because there may not be other (additional males) coming in her vicinity, and mating is probably better than remaining unmated. Interestingly, while theory on non-signalling females predicts that virgins should be less choosy than mated females (Jennions & Petrie, 2000; Kokko & Mappes, 2005), theoretical predictions for virgin and mated female reproductive strategies for signalling females are, to our knowledge, lacking. Such predictions are important to understand the evolution of female signalling and choosiness.

In this study, we determined how signalling differs between virgin and mated females and assessed whether predictions from non-signalling females (i.e., that virgins are less choosy than mated females) hold for signalling females. Females of the tobacco budworm Chloridea (Heliothis) virescens (Lepidoptera: Noctuidae) are an ideal study system, because these female moths signal and also choose their mating partners (Zweerus et al., 2021). Also, decades of research on this species provides much information on its mating behaviour. Females signal by evertting their pheromone gland from the tip of the abdomen, a behaviour termed ‘calling’, which releases the species-specific sex pheromone to attract males (Tumlinson et al., 1975; Löfstedt, 1993). Female signalling and male responses are synchronized within a specific window of time at night (reviewed in Phelan, 1997; Groot, 2014). Under laboratory conditions, C. virescens females start signalling about half-way into the scotophase (i.e., the dark period of the diel cycle), peaking at around 6 hours into the dark under 14:10 L:D conditions (Heath et al., 1991; Pope et al., 1982; NLZ pers. observation). Both males and females mate only once per night, but several times over multiple nights (Flint & Kressin, 1968; Raulston et al., 1975; Pair et al., 1977; Gao et al., 2020). Previous research showed that females choose mates during courtship and virgin C. virescens females prefer to mate with larger, higher-quality males (Zweerus et al., 2021). Matings generally last 2-3 hours (Pair et al. 1977; Blanco et al. 2009; Hosseini et al. 2016), during which the male transfers a spermatophore (LaMunyon, 2000; Blanco et al., 2009). Even though one spermatophore can provide enough sperm to inseminate all eggs (LaMunyon, 2000), females mate multiple times (Raulston et al., 1975; Blanco et al., 2009). In multiple-mated C. virescens females, sperm precedence is variable (LaMunyon & Eisner, 1993; LaMunyon, 2000; Blanco et al., 2008). Mated females gain fitness with additional matings as their offspring number increases with every mating, peaking after three matings (Gao et al., 2020).

To test the effect of mating status on sexual behaviour and mate choice, we conducted three experiments on virgin and mated C. virescens females. First, we evaluated whether virgin females and mated females differ in their signalling effort. Since the necessity to mate is higher in virgin females than in mated females, we expected virgin females to signal more or for longer than mated females. Second, we tested the hypothesis that virgin females have a shorter mating latency (as a proxy of readiness to mate) than mated females, again because virgins must secure
a mate. Finally, we tested if virgin females show a weaker preference for larger males than mated females because less stringent selection of males creates a higher chance for mating.

**METHODS**

**Study organism**

*Chloridea (Heliothis) virescens* populations originated from North Carolina State University (YDK strain) and the Max Planck Institute for Chemical Ecology, Jena, and have been reared at the Institute for Biodiversity and Ecosystem and Dynamics (IBED), University of Amsterdam since 2011. We conducted the experiment for this study between April 2018 and November 2019. The moths were kept in a climate chamber at 60% relative humidity and 25 ± 1°C, with a 14 h light (photophase): 10 h dark (scotophase) photoperiod (lights off at 11 a.m. CET). Larvae were reared on artificial pinto bean diet (Burton, 1970) in individual plastic cups (37 ml, Solo, Lake Forest, Illinois). Pupae were checked daily for eclosion (i.e., hatching of adult) and emerged adults were fed 10% sucrose solution provided through 1 cm cotton dental wick. All experiments were conducted with 2–3-day old non-sibling individuals and were conducted under the same environmental conditions as those used for rearing.

**Procedure to obtain mated females and maintaining virgin females**

To obtain mated females, we placed an adult female (first day after eclosion) in a large clear plastic cup (473 ml, Solo) with an adult male that was randomly chosen from the adults of the standard rearing. At the same time, for the ‘virgin group’ females were also selected on the first day after eclosion and individually isolated. We next observed all pairs at 30 min intervals during the scotophase (dark period) until all pairs were mating, or until 9 hours of the scotophase had passed. Because *C. virescens* matings last for several hours, only females that mated for ≥ 60 min qualified for the ‘mated group’ of the experiment. In the photophase that followed the scotophase (i.e., light period on day 2), we individually isolated the mated females. We conducted the experiments on the third day after eclosion, under the same environmental conditions as the rearing and the preparatory matings.

**Procedure to obtain an extended range of male body sizes**

Previously we found that male body size affects female fitness: females mating with larger males had more offspring than when mating with smaller males (Zweerus et al., 2021). To assess female preference for males of different body sizes (a proxy for quality), we increased the range of male sizes by altering the larval diet, following Zweerus et al. (2021). Briefly, we obtained males of average to large size by rearing larvae on a standard pinto bean diet (Burton, 1970), and obtained males of smaller than average size by rearing larvae on diet which nutritional value was 25% of the standard diet. It is important to note that even on standard diet, male mass varies. The low-nutritional diet extended this ‘natural’ range of masses at the lower end (see Zweerus et al., 2021, Fig. S1). The diet treatment was thus a tool to increase the range of male masses without being a factor in the experimental design. For the experiments, we did not use males from one or the other diet, but rather males that differed in pupal mass.
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**Mate-attraction effort experiment**

To compare mate attraction between virgin and mated females using their signalling behaviour as a proxy of mate attraction effort, we quantified the signalling activity of all females (virgin: \(n = 24\) and mated: \(n = 21\)) in the third scotophase after eclosion as follows. In the hour prior to the scotophase, we placed single females into large clear plastic cups (473 ml, Solo). From the onset of scotophase, we observed and scored the number of females signalling every 15 minutes. We stopped observing an individual if the female did not signal for at least 1 hour. To test if the proportion of signalling females differed between the virgin and mated group, we used a Chi-square test for independence. To assess if virgin females (\(n = 21\)) started signalling later than mated females (\(n = 17\)), we analyzed the normally-distributed data with equal variances for the onset time of signalling with a two-tailed \(t\)-test. To test if the duration of signalling differed between virgin and mated females, we used a Mann-Whitney-\(U\)-test, because the data were non-normally distributed (assessed by Shapiro-Wilk test and visual exploration of histograms). We visualized the data by fitting the proportion of signalling females per time point and mating status over the scotophase using the package ggplot2 (Wickham, 2016) in the software R (version 4.0.5, R Core Team (2021)).

**No-choice experiment to assess readiness to mate in virgin and mated females**

To test the hypothesis that virgin females have a shorter mating latency (as a proxy of readiness to mate) than mated females, we measured their mating latency (the time from the pairing until copulation) in no-choice mating assays. Firstly, we placed one female with one male into a clear plastic cup (473 ml, Solo) and covered the cup with a mesh. We then mounted each plastic cup in a hanging grid with a camera (GoPro Hero silver) underneath. The assay started at the beginning of the scotophase, after which we recorded a time-lapse series of 1pic / min. We collected the data on minimally 4 and maximally 20 samples per time over 8 scotophases between 11\(^{th}\) and 20\(^{th}\) of May 2018. To determine mating latency, we identified the timestamp of the picture showing a newly-formed mating pair in the time-lapse series. For individuals that did not mate, we censored their data by assigning a maximum time span of 600 min, which corresponds to an entire scotophase. To test if virgin females (\(n = 61\)) mated significantly earlier than mated females (\(n = 29\)), and whether male pupal mass or female pupal mass affected mating latency, we fitted a Cox proportional-hazards model with the explanatory variables mating status, male pupal mass, and female pupal mass as main effects and mating latency as the response variable in R using the packages survival (Therneau, 2021), and survminer (Kassambara et al., 2019).

**Two-choice experiment to compare virgin and mated female mate preferences**

To test the hypothesis that virgin females show a weaker preference for larger (i.e., higher-quality) males than mated females, we conducted two-choice tests, in which we placed one female (virgin: \(n = 61\) or mated: \(n = 63\)) together with a larger and a smaller male into a BugDorm cage (H30 cm x W30 cm x D30 cm), and scored which male a female mated. To distinguish between the two males, we marked each male by clipping the tip of one randomly chosen wing. The experiment started 10 min before the onset of the scotophase and we checked all cages at
30 min intervals. Once a mating pair had formed, we removed the unmated male from the cage. To check if the matings were successful, we isolated and froze all individuals in the next photophase, dissected the females, and quantified the number of spermatophores per individual. We collected the data over a total of 13 scotophases between 13th Augustus and 8th November 2019. Since not all females mated in the two-choice assay (virgin: n = 56, mated: n = 45), we first tested for an association between mating status (virgin, mated) and mating occurrence (mating, no mating) with a Fisher’s exact test. Additionally, we confirmed that the mass range of larger and smaller males did not differ significantly between males offered to virgin females and to mated females, using a Welch’s two-sample t-test. Finally, we determined if the size range between virgin females and mated females differed using a Welch’s two-sample t-test.

To assess whether the males that females chose to mate with were on average larger than the rejected males, we first tested if mean pupal mass differed significantly between the chosen and not chosen males by computing paired t-tests. To then test whether virgin and mated females differed in the strength of their mate preference, we first randomly selected the data of one male per cage (i.e., this male was either chosen or not chosen by the female). Since each female made one choice, this step ensured that the number of data points for the analysis corresponded to the actual number of choices made in the experiment. Subsequently, we modelled the response variable female choice as a function of the variable larger/smaller (male), indicating whether the male was larger or smaller compared to the other male in the same cage, the difference in male pupal mass between the two males per cage, female mating status, and their three-way interaction. Including a three-way interaction in the model allowed us to let the slopes of the function vary independently, and thus enabled us to identify the differences in female mate choice with respect to female mating status. We fitted the model (glm) with a binomial error distribution and produced the ANOVA table using the package car (Fox & Weisberg, 2019) in R. The results were visualized using the package ggplot2 (Wickham, 2016).
RESULTS
Mate-attraction effort experiment: virgin females signal more, but not earlier, than mated females
There was no significant difference in the proportion of virgin and mated females that signalled in the third scotophase after eclosion (virgin: n = 21, 87.5% (21/24), mated: n = 17, 80.9% (17/21); \( \chi^2_1 = 0, P = 1.00 \)). Also, both virgin and mated females started signalling around the same time into the scotophase (virgins: 291.4 ± 14.4 min SE into scotophase, mated: 298.2 ± 21.9 min, t-test: \( t = 0.269, df = 36, P = 0.797 \) (Fig. 1a). However, virgin females spent more time signalling (90 min, IQR 75 – 150 min) than mated females (45 min, IQR 15 – 120 min) (U = 476.00, \( P = 0.049 \)) (Fig. 1b). Overall, more virgin than mated females signalled per time point (Fig. 1c).

No-choice experiment: virgin and mated females are equally ready to mate
In the mating latency assay, 86 out of 90 pairs (61 virgins and 29 mated females) mated, while three virgin females and one mated female did not mate. Neither male mass (\( \chi^2_1 = 0.49, P = 0.48 \)) nor female mass (\( \chi^2_1 = 2.3, P = 0.13 \)) significantly affected mating latency. In contrast to our hypothesis, virgin females did not mate significantly quicker than mated females (log-rank test:}
\( \chi^2_1 = 2.9, P = 0.09 \) (Fig. 2). Interestingly, however, there was a non-significant trend towards the predicted direction.

Fig. 2. Mating latencies of virgin (in grey) and mated females (in blue), expressed as survival curves over time. Shaded area around each curve: 95% confidence interval. Dashed lines: time point when 50% of the females mated.

**Two-choice experiment: virgin females are less choosy than mated females**

The ranges of male mass did not significantly differ between the trials involving virgins (132.1 - 292.0 mg, with a mean of 225.4 ± 3.5 mg SE) and mated (128.6 - 316.4 mg, with a mean of 224.4 ± 4.5 mg) females (Welch’s t-test: \( t = -1.175, df = 177.24, P = 0.861 \)) (Fig. 3a and Fig. 3b). In the two-choice experiments, 91.8% (56/61) of virgin females mated, while only 71.4% (45/63) of mated females mated (Fisher’s exact test, \( P = 0.014 \)) (Fig. 3c). Males chosen by virgin females weighed on average 229.4 ± 4.5 mg and the non-chosen males averaged 221.4 ± 5.4 mg (paired t-test: \( t = -0.958, df = 55, P = 0.343 \)) (Fig. 3a). In comparison, males chosen by mated females weighed on average 238.0 ± 4.9 mg, while the non-chosen males had an average pupal mass of 210.8 ± 7.0 mg (paired t-test: \( t = -3.152, df = 44, P = 0.003 \)) (Fig. 3b). Post-hoc analysis on female size showed that the size range of virgin females (\( n = 56 \)) was 115.2 - 311.6 mg (mean 223.9 ± 5.4 mg SE) and similar to the size range of mated females (\( n = 45 \)), which was 148.0 - 293.5 mg (mean 226.7 ± 5.3 mg SE) (t-test: \( t = 0.367, df = 99, P = 0.714 \)).
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Mated females selected more strongly for relatively larger males (Fig. 4). In testing the strength of female preference in virgin and mated females, we found a highly significant, three-way interaction in our model ($\chi^2_1 = 11.244$, $P = 0.004$, Table 1), showing that virgin females ($n = 56$) had a weaker preference than mated females ($n = 45$) (Fig. 4). This result thus confirms the hypothesis that virgin females are less choosy than mated females. Moreover, our model revealed that the relative male mass significantly affected female choice in mated females ($\chi^2_1 = 4.486$, $P = 0.034$, Table 1). Once the mass difference between the two offered males exceeded 55 mg, mated females differentiated between larger and smaller males, while virgin females did not (Fig. 4).

Fig. 4. Probability that a virgin (left panel) or mated female (right panel) chooses the relatively larger (red) or smaller male (blue) offered to the female. The curves show model predicted values for male mating probability, based on the difference in male mass. Jittered dots: actual data points. Dashed line: 50% mating probability. Shaded area around curves is the 95% CI.
Table 1. Analysis of Deviance Table. Model structure: choice ~ (larger/smaller x mass difference) x mating status + larger/smaller + mass difference + mating status

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DISCUSSION

In this study, we compared female sexual behaviour and mate choice between virgin and mated females in a system where females are not only choosers but also signallers. As expected, we found virgin females to signal longer than mated females. We also found that virgin and mated females showed similar mating latencies, our proxy for readiness to mate. When females could choose between two males, virgin females showed a weaker preference for larger males compared to mated females. Below we discuss these findings in the context of multiple matings.

Signalling behaviour

Since virgin females must reduce the risk of remaining unmated, we hypothesized virgin females to signal more or for longer than mated females, which we confirmed in this study. A higher signalling rate of virgin compared to mated females has previously been found in other Heliothine moths, such as the corn earworm *Helicoverpa zea* (Kingan et al., 1993), the subflexus straw *Chloridea (Heliotis) subflexa* (Blankers et al., 2021), and the Pyralid Indian meal moth *Plodia interpunctella* (Brady & Smithwick, 1968). These intuitive findings support the notion that virgin signalling females put more effort into mate attraction effort compared to mated females to increase their chance for mating.

Female mating status had no effect on the onset time of signalling, and we found virgins and mated females to signal within the same time window. Finding overlapping female signalling windows is consistent with species-specific synchronized signalling, which minimizes communication interference with individuals from closely related species (Teal et al., 1978; Pashley et al., 1992; Monti et al., 1995; Schöfl et al., 2009; reviewed in Groot, 2014). Even though mated females would have obtained enough sperm from the first mating to fertilize all their eggs (LaMunyon, 2000), we found that about 30% of mated females continued to signal in the night that followed a mating. In the closely related moth *C. subflexa*, a lower percentage of females was found calling in the night after mating, however, calling effort increased to about 50% again in subsequent nights (Blankers et al., 2021). The fact that mated females continue to signal emphasizes that both sexes may benefit from mating multiply (Gao et al., 2020; Blankers et al., 2021).

Female readiness to mate (i.e., mating latency)

We found no difference in mating latency between virgin and mated signalling females and thus their readiness to mate, which contrasts the hypothesis that virgin females mate quicker than
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Mated females (Kokko & Mappes, 2005). While a shorter mating latency would be expected in non-signalling and non-choosy virgin females, our results suggest that signalling virgin females might be choosy. As females were only offered one male (no-choice assay), the mating latencies that we measured are possibly the minimal times that females need for mate assessment. Female mate assessment likely starts after 120 min into scotophase, because both virgin and mated females only start become sexually active after this time point, as reflected by their signalling behaviour (see Figure 1). It must be noted here that context does affect the timing of sexual activity, as female sexual activity started earlier when a male was present than when females were alone in the signalling assay, even though the cups used in these experiments were identical. Matings occurred throughout the entire period in which females signalled. The fact that we found virgin and mated signalling females equally ready to mate further makes sense, because multiple matings increases female fitness in this species (Gao et al., 2020).

**Female mate preference**

In line with the hypothesis that virgin females have a weaker mate preference than mated females, we found that virgin females had a weaker preference for larger males than mated females. This finding is consistent with predictions for non-signalling females where virgins are expected to be less choosy (Kokko & Mappes, 2005). However, these predictions did not take into account that signalling may result in attracting multiple males. Apparently, signalling is a factor that allows virgin females to be choosy, although not to the same extent as mated females, which may be due to the fact that virgin females are constrained by their necessity to mate. Once a first mating has been secured, *C. virescens* females can increase their fitness by stronger selection for high quality males.

Whereas the most obvious effect of the reduced diet was a reduction in male pupal mass, other effects of the reduced diet on male phenotypes cannot be excluded. In our experiments we manipulated the difference in body mass, because we already discovered that this variable affects female choice (Zweerus et al., 2021). However, we cannot exclude that diet affected additional traits unknown to us that contributed to female choice.

**Future perspectives: mate choice of polygamous females**

Recently, Gao et al. (2020) discovered that *C. virescens* females reach maximal fitness with three matings, which suggests that females should maintain a (low) level of choosiness to increase their mating chances until they have reached their mating ‘optimum’ (Gao et al., 2020). However, because female fitness declines after three matings (Gao et al., 2020), thrice-mated females may become choosier, potentially to such an extent that these females will not mate if no male meets their mate acceptance threshold (De Jong & Sabelis, 1991). To unravel how females optimize their fitness, it would thus be interesting to assess whether mate preferences become stronger when females mate more than twice, and how sequential mate choice translates into paternity (see also Slatyer et al., 2012; Kokko et al., 2006).

Since *C. virescens* females mate multiply to increase fitness, mated females may “trade-up” in partner quality when they remate (Halliday, 1983; Jennions & Petrie, 2000). Such a trading up further increases (genetic) benefits and maximizes fitness. For example, in guppies, females...
not only remate with higher quality males, but their eggs are also more likely to be fertilized with sperm from the higher-quality male (Pitcher et al., 2003). In *C. virescens*, females can perhaps bias paternity towards higher-quality males to increase the genetic benefits for their offspring. The fact that sperm precedence in *C. virescens* is variable (LaMunyon, 2000, 2001) suggests that cryptic female choice may occur in this species.

**Female mate choice in signalling females compared to non-signalling females**

In conclusion, our results show that in a species where females are signallers as well as choosers, virgin females are choosy too. This differs from the general idea that virgin females mate unselectively, which may be the case in species without signalling females. Non-signalling females cannot affect mate availability, and virgin females are probably more likely to mate with any available male and thus less choosy than mated females to decrease the risk of remaining unmated. In contrast, signalling allows virgin females to be choosy, because signalling females can affect the arrival and number of available males. Since multiple males may be attracted, both virgin and mated females likely benefit from choosing the best male. This explains our finding that virgin and mated females were equally ready to mate. We propose that female signalling should be considered as a crucial component of female mate choice and taken into account to understand the evolution of female choice.

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