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One and Done: Long-Term Sperm Storage in the Cactus-Feeding Bug, *Narnia femorata* (Hemiptera: Coreidae)

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Abstract

Female sperm storage is common across a wide taxonomic range. The temporal separation of mating and fertilization has several benefits for females. It enables sperm selection from multiple males, but can also allow females to temporally and spatially delay fertilization until the proper environmental conditions are found. In this study, we investigated the extent and possible function of sperm storage in the polygamous cactus-feeding bug, *Narnia femorata* Stål (Hemiptera: Coreidae). To determine the viability of stored sperm over time, we tracked life-long fecundity of females exposed to varying levels of male access. We exposed females to four treatments: one male for 1 wk, one male for 1 wk with further exposure to the same male later in her life for an additional week, one male for the duration of her life, or two males (subsequent) for the duration of her life. Our results indicate that females can store sperm and produce viable offspring during their lifespan from a relatively brief mating encounter with a single male. Furthermore, egg production and fertility rates did not differ across treatments, suggesting that time of exposure to mates and number of mates (monandry vs. polyandry) has no effect on *N. femorata* fecundity. Sperm storage seems to operate independently of mate number or availability, and is therefore, likely an adaptation to the patchy spatial-temporal distribution of adequate resources.

Key words: hatching success, leaf-footed bug, multiple mating, polygamy, spermatheca

A myriad of organisms have the capacity to provide a suitable environment within the female reproductive tract to sustain viable sperm long after mating, referred to as sperm storage (Birkhead and Møller 1993, Neubaum and Wolfner 1999, Orr and Zuk 2012, Orr and Brennan 2015). In insects, sperm from a single or multiple males can be stored in specialized organs called spermathecae, allowing for differential storage and use (Lewis and Jutkiewicz 1998, Hellriegel and Bernasconi 2000, Bretman et al. 2009, Orr and Brennan 2015). For example, queen honeybees (Collins et al. 2006) and leaf-cutter ants (den Boer et al. 2009) can store sperm throughout their lifetime (over multiple decades in some ant species), which allows females to decouple copulation and fertilization, and at the same time may allow post-copulatory sexual selection to take place (Baer 2005, McCullough et al. 2017). Thus, long-term sperm storage can be especially advantageous in mating systems involving polyandry—which is a pattern of mating where a female mates with multiple males—if it is in the female's best interest to be choosy regarding sperm (Eberhard 1996). Nonetheless, even with our current understanding of sperm storage, there are still many questions regarding the selective forces behind its evolution (Orr and Brennan 2015).

The ability to produce offspring even when few males are readily available for reproduction has been hypothesized as one of the main benefits of sperm storage. The decoupling of mating and fertilization may be important due to factors like migration or large home ranges (Orr and Zuk 2012), sex-specific mortality (Wiklund et al. 2003), and sexual cannibalism (Herberstein et al. 2011), allowing females to fertilize eggs when males are no longer around. Furthermore, the spatial and temporal separation of copulation and fertilization can allow for fertilization and offspring production to occur only when environmental conditions are most suitable, e.g., when food availability and temperature are preferred (Walker and Nation 1982).

Sperm storage can also provide another arena for sexual selection to occur if females mate with multiple males. Females can potentially copulate with many mates in a short period, store the sperm (Collins et al. 2006, den Boer et al. 2009), and then select which sperm to use throughout their lifetime (Baer 2005, McCullough

1

et al. 2017). Thus, despite potential costs of polyandry, like time and energy loss (Watson et al. 1998), increased predation risk (Arnqvist 1989, Rowe 1994), and disease (Knell and Webberley 2004), which may reduce female egg production rates (Arnqvist and Nilsson 2000), polyandry can provide females with several direct and indirect benefits (reviewed in Arnqvist and Nilsson 2000). Mating with multiple males may increase a female's fitness by promoting post-copulatory sexual selection mechanisms such as sperm competition, which favors paternity towards viable and/or genetically compatible sperm (Zeh and Zeh 1997; Tregenza and Wedell 1998, 2000; Jennions and Petrie 2000), including the avoidance of inbreeding (Tregenza and Wedell 2002). Therefore, post-copulatory sexual selection has an undeniable influence on the genetic composition of populations across taxonomic groups (Fedorka and Mousseau 2002, Calsbeek et al. 2007, McCullough et al. 2017). Hence, the duration that viable sperm can be stored may indicate the underlying mechanisms behind mating systems and reproductive success; e.g., males of polyandrous species of insects have stored sperm that lasts longer than in males of monandrous species (Hunter and Birkhead 2002).

To better understand female sperm storage potential, we used the leaf-footed cactus bug, *Narnia femorata* Stål (Hemiptera: Coreidae). These bugs are commonly found in the southwestern United States, Mexico, and Florida (Baranowski and Slater 1986), where they feed on several species of opuntioid cacti (Vessels et al. 2013). Female cactus bugs possess spermathecae, which can store sperm (Sasson et al. 2016), but the fitness consequences of this process are not well understood. Both sexes have the opportunity to mate with many partners during their lifetime. Males of *N. femorata* use their enlarged hind femurs as weapons (Procter et al. 2012, Nolen et al. 2017) as they defend cactus territories awaiting female visits. Females do not hold territories, they deposit their eggs on the cactus spines soon after mating or they can leave and deposit them somewhere else. Therefore, females likely mate with multiple males, feeding and laying eggs in several locations. Under laboratory conditions, *N. femorata* females will mate several times with the same male over a 3-h period (Cirino and Miller, unpublished data) but also over several weeks (P. E. A., personal observation). Females will also mate with multiple males in succession over a 2-h period (Emberts et al., 2018). Importantly, healthy virgin females are ready to lay eggs as soon as they become sexually mature (12–14 d after molt), and will start scattering/discarding unfertilized eggs until they mate. Therefore, early mating is necessary to avoid loss of the nutritional and energetic investment of egg production.

In this study, we explored the practical capabilities of sperm storage in *N. femorata* by investigating lifetime egg production and fertilization rates. Our two main objectives were to determine: 1) How long *N. femorata* females can store viable sperm and 2) If the number of males a female mates with influences egg production and/or hatching success. We predicted that access to males for a longer period would increase egg production and hatching success, as a consequence of sperm depletion in isolated females. Regarding our predictions of one versus two mates, the empirical evidence is mixed (Arnqvist and Nilsson 2000, Jennions et al. 2007), but we assume that mating with multiple males is beneficial in polyandric mating systems (McCullough et al. 2018) even if there is an associated cost (e.g., higher risk of injury or death; Worthington and Kelly 2016). We predicted that mating with a second male would encourage (e.g., due to increased genetic diversity of offspring; Gershman 2009) the females to either lay more eggs, fertilize more eggs, or both, and therefore increase fecundity.

Materials and Methods

Insect Collection and Laboratory Rearing

Fourth and fifth instar *N. femorata* nymphs were collected from the Ordway Swisher Biological Station in Melrose, FL (29°41′N, 82°W), and immediately transported to a laboratory at the University of Florida campus, Gainesville, FL; during late October and early November 2013. Insects were housed individually in plastic deli cups (secured by a mesh lid), with a cactus pad (*Opuntia mesacantha* Small (Caryophyllales: Cactaceae)) planted in a 2.5 cm layer of topsoil and maintained in a greenhouse (25–32°C daily temperature range). All cups were supplemented with ripe cactus fruits (*O. mesacantha*), which were replaced as needed (e.g., desiccation or decay). After the insects reached sexual maturity (2 wk after the final molt) females were randomly assigned to one of four treatments (see Experimental Design and Treatments), and males were randomly assigned to each female. *N. femorata* females usually lay their eggs on the spines of the cacti. We removed the spines and added pine needles, which served as an ideal egg laying substrate that was easily extracted from the experimental cups. Mating pairs were placed into the cups and into an incubator held at 26°C, 80% humidity, and a 14:10 (L:D) h cycle.

Experimental Design and Treatments

Four treatments were used to test the duration of stored sperm viability inside the female (or alternatively, sperm depletion), and the influence of mate number on egg production and hatching success. The first treatment, coded 'Male for a week', consisted of a male and a female enclosed together for the first week after sexual maturity was achieved. We then removed the male and left the female isolated throughout the entire duration of the experiment (when the female died or when the experiment ended [week 21]; see Data Collection section). The second treatment, coded 'Male for a week + conjugal', consisted of a male and a female enclosed together for the first week, removing the male for 8 wk, reintroducing the same male for 1 wk, and finally removing it for the rest of the experiment. These males were kept separately and isolated in cups with cactus and fruit. Up until the ninth week, this treatment is equal to 'Male for a week'. The third treatment, coded 'One male', consisted of a male added into the experimental cup with the female and left for the entire duration of the experiment. In the fourth treatment, coded 'Two males', the female and a male were left together for 4 wk, then the male was randomly switched with another male from the same treatment and left for the remainder of the experiment. Up until, and including, the fourth week, this treatment is equal to *One male*. All treatments consisted of 11 experimental cups, except for the 'Male for a week' treatment, which only had 10 cups. The 43 initial males were placed in the experimental cups at the same time. We used 1 wk as our shortest contact period between mating pairs as not all pairs mate readily within the first 24 h of pairing (Joseph et al. 2017); meanwhile, our extended experience with this organism indicated that within a week all pairs would mate at least once.

The 'Male for a week' treatment was used to measure sperm storage viability and egg production from a single, brief encounter with a male. The 'Male for a week + conjugal' treatment was used to test if the reintroduction of a male influenced egg production and/or fertility rates. If sperm depletion occurred, this treatment would have provided evidence of replenishment. The 'One male' treatment was used as the control for the sperm storage viability aspect, to measure the effect of a constant male presence (source of sperm) on egg production and hatching success. The 'One male' treatment also acted as our lifetime monandry treatment, while the 'Two males' treatment

simulated polyandry (Tregenza and Wedell 1998, Worthington and Kelly 2016). With these last two treatments, we were able to determine if the number of males a female mated with influenced egg production and/or hatching success.

Data Collection

We carried out the study for 21 wk at which point only 21 of the original 43 females were still alive, and only three females were still producing eggs. Egg production was determined by counting all eggs produced by each female on a weekly basis. We removed and replaced pine needles weekly. Pine needles with eggs were kept individually inside clear plastic containers in the incubator; pine needles were kept in the same orientation as in the deli cups, as eggs do not always hatch if their orientation is changed. We began checking for successful egg hatch in the third week of the experiment, after 14 d of eggs being laid, by counting the number of hatched eggs and number of nymphs (redundancy for accuracy) in the separate plastic containers. Adult survivorship was also monitored on a weekly basis.

Female body size may influence sperm storage ability (Arnqvist and Nilsson 2000) and egg production (Miller et al. 2013). Therefore, after week 21, females were frozen (individuals that died earlier were frozen immediately) and images were taken with a digital camera (Canon EOS 50D) attached to a dissecting microscope (Leica M165 C). We used ImageJ image analysis software (Schneider et al. 2012) to obtain a linear measurement of pronotum width, which predicts overall body size (Procter et al. 2012, Gillespie et al. 2014, Allen and Miller 2017).

Data Analysis

All analyses were conducted with IBM SPSS ver. 24. We first tested if body size, as we measured it here, had a significant effect on total egg production. To do this analysis, we treated male access (four treatments) as our fixed factor, body size as a predictor co-variable, and total egg number (after 15 wk, see General Patterns section of Results) as the response variable in a simple Analysis of Covariance (ANCOVA). Because 50% of the total number of eggs was produced during the first 4 wk, we ran a similar ANCOVA, but limited to only those weeks and only the two treatments in effect at that point ('Male for a week' vs. 'One male'). We found no relationship

between these traits in either analysis (see Egg Production), thus we did not include body size as a covariate in subsequent analyses. We then proceeded to use repeated-measures Analysis of Variance (ANOVA) to determine if differences in male availability (access to a male after 1 wk; lifetime access to one or two males) affected egg production and hatching success (number of hatched nymphs) over the entire experiment period, per week.

Instead of analyzing fertility rates as proportions (hatched nymphs/eggs produced), we used number of offspring produced (nymphs hatched), as we did not find significant differences in egg production across treatments (see Egg Production), but more importantly because the use of proportions (or ratios) in this kind of analysis is statically inappropriate (Warton and Hui 2011). Furthermore, we are interested in both absolute offspring output (total number of nymphs) and egg viability, as they reflect different fitness components (Note: the hatching rate for week 1 was not included in any analyses; due to a logistical error only 16/43 nymph totals were checked, four per treatment. However, there is no reason to believe these data would change the outcome of the analysis, mean hatching rates [$n = 4$ per treatment] for week 1 ranged from 0.62 ± 0.21 to 0.98 ± 0.03). Additionally, using all the complete data (week 2–15), we analyzed fertility rates across treatments using a binomial Generalized Linear Model (GLM), where each of a female's hatched eggs were treated as 'successes = 1' and unhatched eggs as 'failures = 0' in a binomial process. Treatment and Week were considered fixed factors and female ID was nested within Treatment, we also tested for a Treatment × Week interaction. This analysis strengthens our previous results by directly comparing hatching rates across treatments.

Results

General Patterns

The number of unfertilized eggs produced by all females before being paired was trivial ($n < 20$). All females started laying viable eggs by week 2 at the latest, except for two females in the 'Two Male' treatment who started in week 3. This means that all females had procured sperm by the end of week 3. We did not strictly quantify mating behavior (e.g., frequency), but all the females were observed mating during the first week and also with all the males that they
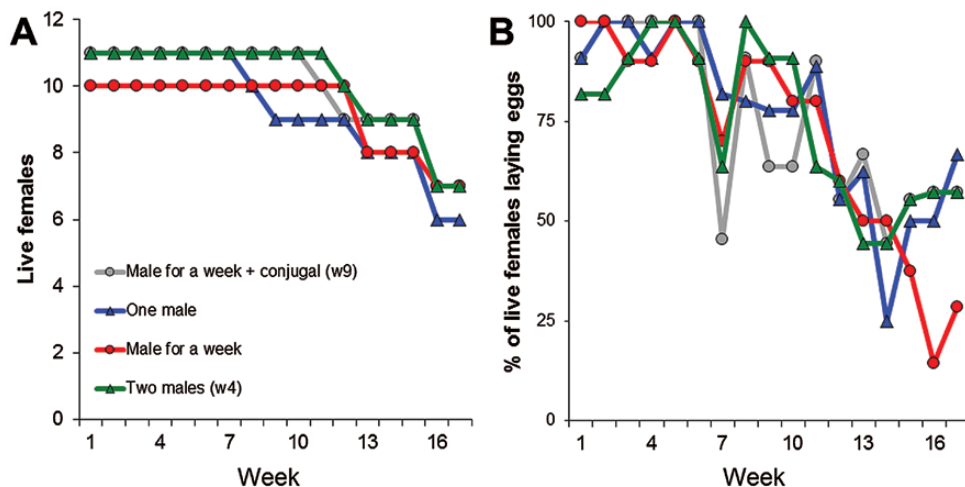

Fig. 1. (A) Total number of live females per treatment, by week. (B) Weekly percent of live females laying eggs per treatment. In the treatments represented by circles (grey/red) the males were left with the female only for the first week and then removed; in the treatment represented by grey circles the male was returned to the same female for 1 wk (week 9) and then removed again. In the treatments represented by triangles (blue/green) the females cohabitated with at least one male for their whole life. In the '*One male*' treatment, blue triangles, females spent their whole life with the same male. In the green triangles treatment, '*Two males*', the male was switched for another male from the same treatment at the end of week 4 and then left there until the end of the experiment.

were exposed to. Additionally, pairs in all possible treatments were observed mating throughout the experiment. By the end of week 21, females had produced a total of 8,557 eggs. On average, an individual female laid 199.0 (SD = 80.0, range: 29–357) eggs during her lifetime. By week 15, females had produced 8,313 eggs, which represent 97.5% of the total amount of eggs produced by week 21. Furthermore, by the end of week 15, all treatments lost at least one female (due to death) (Fig. 1A) and the number of live females laying eggs rapidly dropped from an average of 80% in week 11 to 50% across treatments (Fig. 1B). Therefore, we used data through week 15 for all analyses.

Egg Production
We found no effect of female body size on egg production in neither lifetime (15 wk) nor the 4-wk time periods when female body size was considered (ANCOVAs, Fig. 2). We therefore proceeded without body size as a co-variable in further analyses. The egg-laying pattern was similar between treatments across time, with no significant difference in mean egg production (repeated-measures ANOVA, Table 1, Fig. 3A and B). There was a significant effect of time on egg production (Fig. 3B), with no observed interaction between time and treatment (Table 1). Across time, there was a peak in weekly egg production in week 3 (Fig. 3A and B), and a significant dip in week 7 (Fig. 3B), which coincides with a dip in number of females laying eggs (Fig. 1B). By the end of the eighth week, 80% of eggs had been laid (Fig. 3A and B).

Nymph Viability (Hatching Success)
Because there was no difference in egg production between treatments, we first compared weekly nymph viability using absolute values of number of hatching nymphs instead of hatching rates. Nymph viability closely matched the egg production pattern across treatments, with weekly fertility rates staying high (>80%) in all treatments throughout the largest egg producing period, the first 10 wk (Fig. 3). There was no significant difference in mean nymph hatching success between treatments (Table 1, Fig. 3C and D). However, there was a significant effect of time on number of nymphs hatched as a direct result of fewer eggs being laid as time progressed (Fig. 3C), but with no interaction between time and treatment (Table 1). Total

nymph viability (hatching rate; week 2–15) was also unaffected by male presence treatment (GLM: Wald X^2 = 1.652, df = 3, P = 0.650; Fig. 3D). Nonetheless, we did find significant effects of time (GLM: Wald X^2 = 55.2, df = 13, P < 0.0001; Fig. 3C) and female ID (GLM: Wald X^2 = 339.7, df = 39, P < 0.0001; Fig. 4) on egg hatching success, and a significant treatment × time interaction (GLM: Wald X^2 = 257.3, df = 39, P < 0.0001; Fig. 3C).

Discussion

N. femorata females exhibit long-term sperm storage. Our data suggest that a brief mating stint after achieving sexual maturity is enough to secure sperm for a lifetime of offspring, and that male availability later in life has no significant effects on total reproductive output. Furthermore, lifetime cohabitation with two males (back-to-back), instead of just one, had no effect on total number of offspring. Egg laying behavior followed the same pattern across treatments, peaking on the third week, and with the majority (≈80%) of egg production occurring during the first 8 wk (Fig. 3B). Fertility rates (hatched nymphs/eggs) stayed high (>80%) across treatments for the first 10 wk, after which they decreased and became irregular (Fig. 3C). We found no short- or long-term effects of female size (=pronotum width) on egg production.

Cohabitation with a male for a short or long period of time showed no difference in lifetime reproductive output. We predicted that females with access to a male for 1 wk would produce fewer eggs or have lower fertility rates due to sperm depletion. Contrary to our prediction, we found no evidence of differences in sperm depletion rates between short- and long-term access to a male. Constant female–male cohabitation can be beneficial (e.g., egg production) when males feed females (nuptial gifts), but the evidence is mixed when this nuptial feeding is not present (reviewed in Arnqvist and Nilsson 2000). The patterns are highly variable across taxa (Griffiths and Tauber 1942, Mayer and Brazzel 1963, Turnipseed and Rabb 1963, Nilakhe 1977, Mullen 1981, Turner and Anderson 1983, Kasule 1986, Opp and Prokopy 1986, Fox 1993, Tamhankar 1995, Sakurai 1996, Taylor et al. 1998), which suggests that any conclusions about the adaptive significance of sperm storage should be considered in a case-by-case basis. Under experimental conditions,
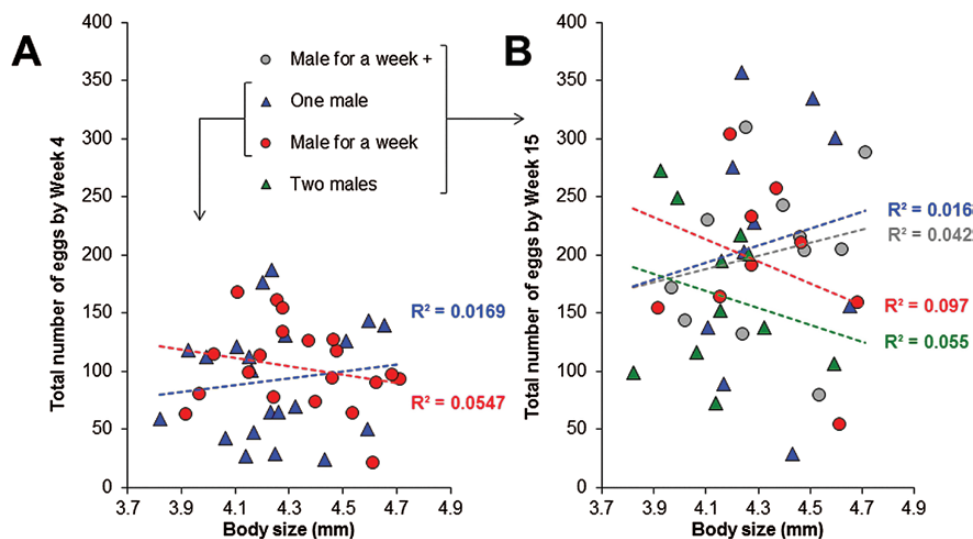

Fig. 2. There was no effect of body size (=pronotum width) in lifetime egg production for *Narnia femorata* females in any of the treatments (male accessibility). (A) Relationship between body size and total egg production during the first 4 wk, when only two of the treatments are in effect (ANCOVA: PW, F = 0.26, df = 1,38, P = 0.87; Treatment, F = 0.63, df = 1,38, P = 0.43). (B) Relationship between body size and lifetime (15 wk) egg production (ANCOVA: PW, F = 0.03, df = 1,37, P = 0.87; Treatment, F = 0.62, df = 3,37, P = 0.60). Females from each treatment are shown in different colors. Regression lines are not significant in all cases (P > 0.3), indicating a lack relationship between body size and egg production. For full descriptions of treatments see text or Fig. 1 legend.

the reduced gains of cohabitation maybe due to the costs associated with injury and harassment (Partridge et al. 1987, Crudgington and Siva-Jothy 2000, Yanagi and Miyatake 2003, Worthington and Kelly 2016). Male *N. femorata* harass and chase females unwilling to mate (L. Cirino, personal communication), however, we found no evidence of any negative effects of prolonged male cohabitation. Additionally, female mortality did not differ across treatments after 15 wk.

We found no difference in either egg production or fertility rates when comparing monandrous versus polyandrous females (Fig. 3). Previous research has shown that when females mate with more males, they will produce more offspring (Tregenza and Wedell 1998, McCullough et al. 2018). Because we did not follow offspring

Table 1. Results of repeated-measures ANOVA examining the effect of male access (presence and/or number) treatment on egg production and hatched nymphs per female.

	df	F	P
Egg production weeks 1–15			
Within-subjects effects			
Time (week)	14,546	36.109	<0.0001
Time × treatment	42,546	0.971	0.525
Between-subjects effect			
Treatment	3,39	0.728	0.541
Nymphs between weeks 2–15			
Within-subjects effects			
Time (week)	13,507	37.377	<0.0001
Time × treatment	39,507	1.312	0.103
Between-subjects effect			
Treatment	3,39	0.604	0.616

development, maturity, and beyond, we cannot make conclusions about female fitness consequences of mating with multiple males beyond the number of offspring. We originally predicted that the exposure to a novel male during peak egg production would have a positive effect at least on offspring production, but egg production was unaffected by treatment conditions and fertilization rates held overall very high across the board (Fig. 3D). Thus, we can conclude that monandry versus polyandry had no effect on total reproductive output under good environmental conditions. This does not mean the polyandry is not beneficial in this species, just that we were unable to detect any benefits.

The direct benefits and costs of polyandry have been documented for several species of insects, even when only using two males as the polyandry treatment (Tregenza and Wedell 1998, Worthington and Kelly 2016). Nonetheless, in other cases, the direct benefits of polyandry are not detected or are minimal (Jennions et al. 2007, Harley et al. 2010), as in this experiment. Regarding the possible indirect benefits of polyandry beyond egg viability, like inbreeding avoidance, increased genetic compatibility, and increased offspring fitness (Ivy and Sakaluk 2005) it was not possible to detect them with our design, and requires further scrutiny (Jennions et al. 2007, Slatyer et al. 2012). Our design also did not include poor environmental conditions that could expose differences between mating treatments. In a separate experiment, we found that under poor nutritional experimental conditions (=immature fruit diet) during adult sexual maturation and adulthood, *N. femorata* egg production was reduced by us as much as 90% when compared to females in similar conditions (=good ripe fruit) as in this experiment (Allen and Miller, unpublished data). Thus, the interaction of environmental factors and different mating treatments could unmask different patterns of offspring production related to both female and male
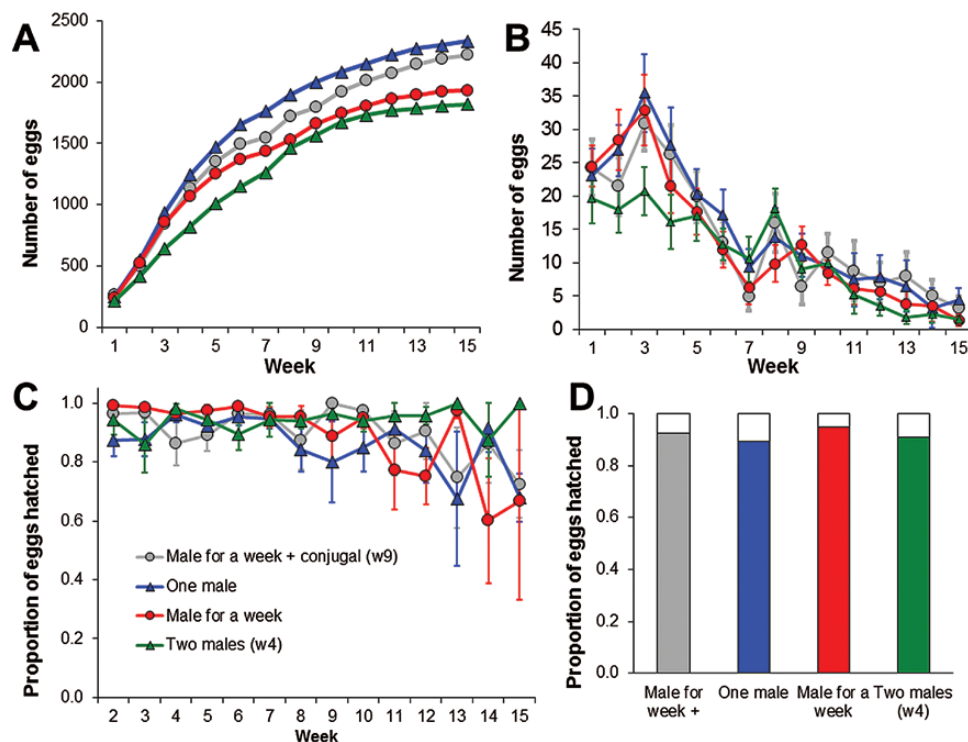

Fig. 3. Egg production and hatching success (=fertility rates) are not affected by male access or number. (A) Cumulative egg production by all females in each treatment. (B) Mean (±SE) number of eggs per female per week, by treatment; means include only live females. (C) Mean (±SE) proportion of eggs hatched per female per week, by treatment; means only include egg-laying females. (D) Percentage of total eggs hatched by treatment from week 2 to week 15. In treatments represented by circles (grey and red) females were paired with a male for only the first week and then males were removed; in treatments represented by triangles (blue/green) females cohabitated with at least one male during their whole lifetime. For full descriptions of treatments see text or Fig. 1 legend.
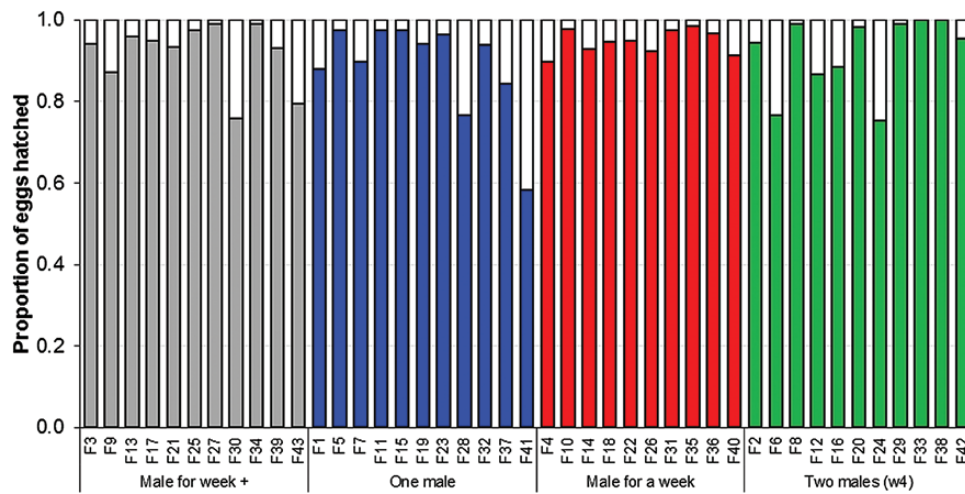
Fig. 4. Percentage of eggs that hatched per female per treatment from week 2 to week 15. Hatching success was significantly affected by female identity. Grey and red treatment females were paired with a male for only the first week and then males were removed; blue and green females cohabitated with at least one male during their whole lifetime. For full descriptions of treatments, see text or Fig. 1 legend.

body condition (Ward 1993, Engqvist and Sauer 2003). Stressful environmental conditions could affect the ability of females to store sperm and also the capacity of males to produce good quality sperm, which could have profound consequences on how females use stored sperm (Ward 1993). Alternatively, it could be that the reproductive resource allocation and associated 'decision making' in this species is a consequence of the anticipation of fluctuating environmental conditions, while polyandry may just provide indirect benefits (Tregenza and Wedell 2002, Ivy and Sakaluk 2005, McNamara et al. 2014).

Long-term sperm storage in *N. femorata* females may be an adaptation to heterogeneous environmental conditions. Females can store a lifetime supply of viable sperm during their first week of sexual maturity, which leads to a lifetime reproductive output that does not differ from that of females that cohabitated with one or two males during their lifetime. The overall pattern seems to indicate that when these females are provided with enough sperm and high-quality food, they will maximize offspring production, no matter male availability or number. In the wild, this species faces several environmental challenges that can make long-term sperm storage a key adaptation. Across their distribution, *N. femorata* feeds of a variety of opuntioid cacti species (Vessels et al. 2013). The cacti fruit are necessary to achieve large body sizes (Gillespie et al. 2014, Miller et al. 2016, Sasson et al. 2016) and for egg production (Allen and Miller, unpublished data), making cactus phenology (e.g., fruit availability) a limiting factor. Females concentrate egg production during the summer when the cacti are flowering and/or producing fruit (Miller et al. 2006, Cirino and Miller 2017), illustrating a natural phenological limitation. Furthermore, depending on local conditions, females also overwinter and start laying eggs as soon as conditions improve (Miller et al. 2006). Thus, long-term sperm storage provides an ideal mechanism to deal with the temporal-spatial uncertainty of both food and male availability.

Further research should combine male availability with environmental heterogeneity to separate the effects of each factor. However, our evidence indicates that access to males after sufficient sperm is stored weighs little on a female's lifetime reproductive output.

Acknowledgments

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