Journal of Evolutionary Biology, 2024, **37**, 905–914
https://doi.org/10.1093/jeb/voae075
Advance access publication 6 June 2024

JOURNAL OF
Evolutionary Biology
eseb

The function of prolonged copulations in *Enchenopa* treehoppers (Hemiptera: Membracidae)

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Handling editor: Rebekah Rogers, Associate editor: Trine Bilde

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Abstract

Copulations are very brief in many species, sometimes taking only seconds, but in other species, they can be quite prolonged. Potential explanations for prolonged copulations include time requirements for the transfer of sperm and/or other ejaculate substances. Ejaculate substances could function to regulate female receptivity to subsequent matings, provide nutritional nuptial gifts, or hasten egg oviposition at a potential survival cost to the female. We investigated prolonged copulation in a member of the *Enchenopa binotata* complex of treehoppers (Hemiptera: Membracidae), in which females rarely remate and copulation can last several hours. We assigned females to treatments in which we interrupted copulation at different times. We also included a control where copulation was not interrupted. We found that females that experienced shorter copulations were more likely to be subsequently receptive to an attractive male. We also found that few females produced offspring when they engaged in short copulations compared to those with longer copulations. We did not find any differences in female survival. Our results support the sperm transfer and receptivity regulation hypotheses. We discuss potential reasons for why these processes should take so long in a species with low female remating.

Keywords: monandry, sexual selection, remating inhibition, accessory substances, Bateman's gradient

Introduction

One of the most widespread patterns in nature involves Darwinian sex roles, whereby males compete for access to females and their eggs, and females selectively accept a subset of the males and their sperm (Andersson, 1994; Bateman, 1948; Darwin, 1871; Janicke et al., 2016; Tonnabel et al., 2019). With Darwinian sex roles, the expectation for copulation duration is based on the notion that reproductive success increases more steeply with the number of copulation partners for males than for females (Bateman, 1948; Janicke et al., 2016; Tonnabel et al., 2019)—although females can also benefit from more copulation partners (Arnqvist & Nilsson, 2000; Fromonteil et al., 2023). Males should, therefore, evolve to quickly mate with numerous females, reducing the time investment per copulation to allow for more time to search, court, and inseminate other females (Alcock, 1994). Copulations across many animal taxa can occur quickly (Amin et al., 2012; Brennan & Orbach, 2020; Bretman et al., 2010; Sultana, 2022). Yet, in numerous other species, copulations are prolonged which increases the total interaction time per mate and restricts opportunities for multiple copulations (e.g., amniotes: Brennan & Orbach [2020]; insects: Brown & Baer [2005] and Carroll [1991]; snakes: Friesen et al. [2017] and Walker & Ford [1996]; mollusks: Ludwig & Walsh [2004]).

There are various reasons why mating pairs may engage in extremely long copulations. Prolonged copulations may be necessary for full sperm transfer either because sperm are transferred gradually and slowly (Campbell & Fairbairn, 2001; Garcia-Gonzalez & Gomendio, 2004; Schneider et al., 2006; Thornhill, 1983) or because a certain interval may be required between intromission and sperm transfer (e.g., if copulatory courtship is necessary for the female to begin to permit sperm transfer) (Eberhard, 1996, 2009). Alternatively, long copulations may be necessary for accessory substances in the ejaculate to be transferred. If the transfer of sperm and accessory substances do not occur simultaneously, then increased copulation time may function to allow sufficient time for either sperm or accessory substances to be transferred. Accessory substances may play a role in the female's regulation of her subsequent receptivity, and perhaps help ensure paternity of the mating male by reducing or inhibiting female remating (Aisenberg & Costa, 2005; Gillott, 2003; Leopold et al., 1971; Murvosh et al., 1964; Simmons & Siva-Jothy, 1998; Yamane, 2013; Yamane et al., 2008). Another possibility is that accessory substances may provide females with a nutritional gift that will help females prolong their lifespan (an indirect method of assessment of this hypothesis) and produce more offspring (Boggs & Gilbert, 1979; Wiklund et al., 1993). Yet another reason for long copulations may be that males pass along stimulants in their ejaculate to induce females to lay eggs sooner and increase the likelihood that their sperm will be used to sire them. This, however, may come at the cost of a reduction in female lifespan (Chapman et al., 1995; Xu & Wang, 2011). Finally, long copulations may be used in mate guarding to physically prevent other males from mating (e.g., using a copulatory plug) (Friesen et al., 2016; Linn et al., 2007).

Received October 4, 2023; revised May 16, 2024; accepted June 4, 2024

The above explanations for prolonged copulations may best apply to animals with polyandrous mating systems where females mate multiply. In such species, there are examples of extreme copulation lengths related to sperm transfer (Campbell & Fairbairn, 2001; Garcia-Gonzalez & Gomendio, 2004; Schneider et al., 2006) or reducing or preventing post-copulatory competition such as sperm competition (Arnqvist & Nilsson, 2000; Kvarnemo & Simmons, 2013). In such cases, the function of long copulations seems clear even though it reduces the time for searching for other mates.

More puzzling is that prolonged copulations also occur in species with low female remating rates (Hughes et al., 2000; Leopold et al., 1971; Orr & Rutowski, 1991). In such species, some of the potential benefits for males who engage in prolonged copulations presumably do not apply (e.g., copulation length may be related to increased sperm transfer but is less likely to be related to sperm competition or cryptic choice). Nevertheless, several other potential benefits from prolonged copulations map apply for species with low remating rates.

We tested four of the five hypotheses described above against each other, modified to fit the case of a mating system where most females only mate once (Table 1). We did not test the mating guarding hypothesis since a requirement of this hypothesis is that females commonly mate multiply. We worked with a member of the *Enchenopa binotata* species complex of treehoppers (Hemiptera: Membracidae) where most females (ca. 95%) mate only once, males can mate more than once, and mating pairs engage in prolonged copulations (Leith et al., 2020; Sullivan-Beckers & Cocroft, 2010; Wood & Guttman, 1982). We experimentally interrupted copulations at different time points. We only allowed females one copulation (as per their mating system). We then assessed female receptivity at two time points after the copulations, quantified egg and offspring production, and tracked female survival. The hypotheses are not mutually exclusive. However, in the framework of the experiment, they each make distinct predictions regarding the effect of copulation interruptions on sexual receptivity, female fecundity, and survival (Table 1).

Methods

Life history, collection, and care

Our study species is a member of the *E. binotata* species complex of treehoppers. When *Enchenopa* females become sexually mature, they engage in substrate-borne vibrational communication with males to find mates (Cocroft & Rodriguez, 2005; Cocroft et al., 2008; Hill, 2008; D. Little, personal communication; Rodríguez & Cocroft, 2006; Rodríguez et al. 2004a, 2006, 2012). Males produce advertisement signals when searching for females. If females find these signals attractive, they respond to them with their own unique signals, and establish a duet with the male. This system of pair formation thus allows *E. binotata* females to decide whether to inform a male about their presence on the plant and allow themselves to be courted. If a female does not respond to a male's signals, the male is not alerted to the female's presence and will fly off the plant rather than search for the female. Duets continue until copulation begins (Figure 1A).

Table 1. Predictions of the hypotheses regarding the function of prolonged copulations in species with a low female remating rate.

Longer compared to shorter copulations should have the following effect on females:			
Hypothesis	**Receptivity**	**Fecundity**	**Adult survival**
Sperm transfer	Lower	Higher	No effect
Receptivity regulation	Lower	No effect	No effect
Nutritional gift	No effect	Higher	Longer
Life history manipulation	No effect	No effect	Shorter

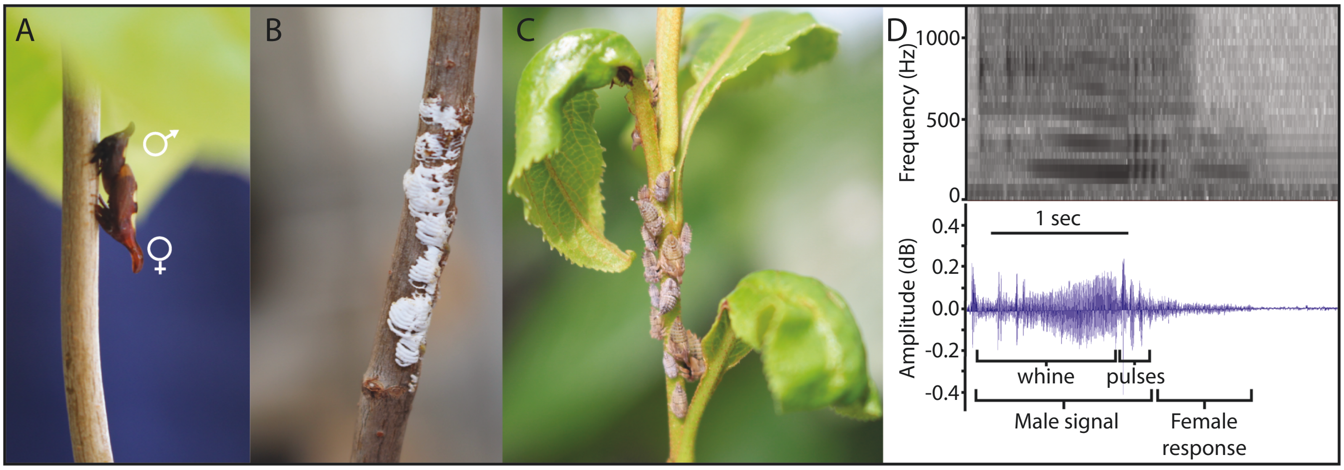

Figure 1. (A) *Enchenopa binotata* treehoppers copulating on their host plant, *Viburnum lentago*. (B) After copulation, one female can lay many egg masses which contain several eggs each. (C) Juveniles developing on the host plant. (D) Sample recording of the male signal playback (used to determine female receptivity) with a female response. This figure shows the spectrogram (top) and waveform (bottom) of the signals. Photo credit: Lauren Cirino.

Courtship and copulation can last up to 6 hr (up to 3 hr for each) (Leith et al., 2020; Sullivan-Beckers & Cocroft, 2010; Wood, 1993) or longer if other males are present (R. Hunt, personal communication). After copulation, females cease responding to male signals (becoming sexually unreceptive) (Sullivan-Beckers & Cocroft, 2010; Wood, 1993; personal observation). Females rarely copulate with an additional male (4% in Sullivan-Beckers & Cocroft [2010] and 5% in Wood & Guttman [1982]) and all offspring produced by a female are likely sired by one male (as is the case in the related treehopper species *Alchisme grossa*: Urquizo et al., 2020).

Females lay eggs in masses (ca. 10 eggs per mass) on their host plants and cover them with a waxy coating (Cocroft et al., 2008; Fowler-Finn et al., 2018). Females can lay between 0 and 140 total eggs in their lifetime, but on average they lay 40 ± 43 (±*SD*) eggs per female (Fowler-Finn et al., 2018). Egg laying begins as early as 1 week after copulation (if copulation occurs late in the breeding season) and females usually lay eggs in late summer through the autumn months (Cocroft et al., 2008). Most males usually die before egg masses are produced (Cocroft et al., 2008; Supplementary Figure 1). Thus, females are the only sex that can provide parental care (Zink, 2003). Females guard egg masses until they die to ensure survival through the autumn and over the long winter (Figure 1B) (Wood, 1993; Zink, 2003). Eggs hatch in the spring when the host plants exit dormancy, leaf out, and sap begins moving through the plant (Figure 1C) (Wood, 1993).

Most members of the *E. binotata* species complex have yet to be formally described (Hamilton & Cocroft, 2009); however, they can be identified by their host plant, juvenile coloration, and dominant male signal (Cocroft et al., 2008, 2010). We used the *E. binotata* species that lives and feeds on nannyberry trees (*Viburnum lentago*, Adoxaceae) in Wisconsin (USA), are grey during the juvenile stage, and have a male dominant- frequency signal of ~165 Hz (Rodríguez et al., 2018).

We collected juvenile treehoppers (second and third instars) in June in southeastern Wisconsin, USA, during two separate field seasons (2021 and 2022) from seven field sites: a portion of the Oak Leaf Trail (43°04′54.2″N, 87°53′26.9″W), Minooka Park (42°58′43.7″N, 88°11′39.1″W), Kletzsch Park (43°8′22.9″N, 87°55′31.8″W), Lion's Den Gorge Nature Preserve (43°20′26.3″N, 87°53′8.1″W), Tendick Nature Park (43°20′26.3″N, 87°53′8.1″W), Warnimont Dog Park (42°55′56.5″N, 87°50′55.2″W), and Waubedonia Park (43°28′6.0″N, 87°58′4.3″W). At each field site, we clipped the ends of branches of several nannyberry trees where aggregates of juveniles usually feed. These nannyberry trees were many meters apart and multiple females congregate to lay and guard their egg masses on the same tree, so each tree likely has numerous genetically diverse offspring (Tallamy & Wood, 1986; Wood, 1974; Zink, 2003). We reared insects on potted nannyberry plants in the University of Wisconsin-Milwaukee (UWM) greenhouse (14:10 L:D, 24.2 ± 3.58 °C). Upon adult eclosion, we separated the bugs by sex to maintain unmated female mating status.

Testing female receptivity

Females become sexually mature approximately 4 weeks after the adult molt (Wood, 1993). Thus, we tested female receptivity prior to the copulation duration trials to ensure females were sexually mature and would engage in signal duetting with a desirable male. We assumed males were already producing signals as they become sexually mature 2 weeks before females (Sullivan-Beckers & Cocroft, 2010). We placed females, one at a time, onto a potted nannyberry recording plant. We played each female a playback of a recorded male signal of the population's mean dominant frequency up to four times to determine her receptivity (six times for two females). We considered females to be sexually mature (i.e., receptive) if they responded to a male signal playback. This is a good assay for female receptivity because once females become receptive ontogenetically, they continue to respond to attractive male signals throughout the mating season until they copulate or die (Cirino et al., 2023; Fowler-Finn & Rodríguez 2013; Speck 2022; Sullivan-Beckers & Cocroft, 2010; Wood, 1993; personal observation).

We used one potted nannyberry plant as our recording plant for all receptivity tests. This recording plant was attached to a piezo-electric stack with accelerometer wax, so that we could transmit the male vibrational signal from a computer (Mac OS X version 10.4.11, Apple, Cupertino, CA, USA). The piezo-controller (MDT694A, Thorlabs, Newton, NJ, USA) regulated the playback from the computer. We focused a portable digital laser vibrometer (Polytec PDV100, Polytec Inc., Irvine, CA, USA) onto a small piece of reflective tape adhered to the recording plant, so we could detect female responses to male signal playbacks. We sent vibrations through a band-pass filter (40–3000 Hz; Krohn-Hite Corporation, Model 3940, Brockton, MA, USA) via a USB audio capture (cakewalk UA-25 EX, Roland Corporation, Hamamatsu, Japan) and recorded on an iMac computer using AUDACITY (version 3.0.2; https://www.audacityteam.org/). We isolated the laser vibrometer setup from building vibrations in multiple ways: (1) We put anti-vibration pads (PneumaticPlus CP6X6, Torrance, CA, USA) beneath the freestanding table legs where the vibrometer and recording plant sat, (2) we placed a ~15-kg epoxy resin tabletop on partially inflated bicycle inner tubes, and (3) we situated the recording plant on a sheet of shock absorbing sorbothane (Edmund Scientifics, Tonawanda, NY, USA). These isolation methods made it so that there was more than 10 dB difference in signal-to-noise ratio. We recorded temperature near the recording plant at the beginning of each receptivity test with a digital thermometer (Extech Instruments Hygro-Thermometer Clock 445702, Twinsburg, OH, USA; temperatures ranged between 22.4 °C and 26 °C ± 0.5).

Copulation duration treatments

We considered females to be receptive and entered them into copulation duration trials if they responded to (duetted with) the above-mentioned playback. Female responses are distinct from male signals and playbacks because they occur shortly after them and have only one element (as opposed to the male whine-pulse structure) and lower dominant frequency (Figure 1D). Thus, this is a clear way to assess whether females are ready to mate with a desirable male. All adults eclosed within 8 days of one another in this experiment. Males become sexually mature 1–2 weeks after adult eclosion (Sullivan-Beckers & Cocroft, 2010), whereas females become sexually mature 3–4 weeks after adult eclosion (Wood, 1993). Thus, males should be signaling once females are sexually receptive. All trees were set up near large windows in the laboratory which allowed sunlight to shine in.

We randomly assigned females to copulation duration treatments of 10, 30, 45, or 60 min. We also included a control treatment where we did not interrupt the copulation. We placed each receptive female onto potted and netted nannyberry trees with a male ($n = 98$ pairs). In two cases, we placed two females on a tree with one male which helps decrease latency to copulation (D. Little, unpublished data). In both cases, we only used the one female that copulated with the male and removed the other female once copulation had started. These two females were in different treatments (10 and 60 min). In total, 98 out of 180 females mated (54.4%) across 35 days of copulation duration trials.

We monitored up to 15 mating pairs simultaneously since it can take anywhere from 2 min to 3 hr for copulations to begin and this wide variation is due, in part, to temperature (Leith et al., 2020). This species is also well suited for concurrent behavior trials because they engage in slow ritualized courtship behaviors that are easy to observe prior to copulation. After finding a female, a male will mount a female dorsoventrally, make genital contact, and, if a female accepts the copulation attempt, the male will proceed with intromission and turn 180° (Figure 1A). We were, therefore, certain to catch the beginning of copulations for all pairings that we set up.

We recorded the initial temperature of the behavior area near the plants using a digital thermometer (Extech Instruments Hygro-Thermometer Clock 445702). Temperatures ranged from 23 °C to 29.1 °C. These initial temperatures may have changed as behavior trials lasted up to 3 hr, but likely only slightly.

If copulation occurred, we separated mating pairs after the designated copulation time by gently grasping the females pronotum and lightly brushing them with a small paintbrush. We then placed females on their own individually potted and netted nannyberry tree to await future receptivity tests, lay eggs, and complete their life cycle. We also kept males individually on netted nannyberry plants.

We used some males (11 out of 84) in multiple behavior trials (10 males used twice and 1 male used four times, treatments randomized). However, we waited at least a week to allow adequate time for ejaculate and sperm stores to replenish (e.g., Greenway et al., 2020; Hughes et al., 2000; Sirot et al., 2009). Females can reject males by not responding to male signals or by not lifting their abdomen to allow intromission (Cocroft et al., 2008; Rodríguez et al. 2004a, D. W. Little & R. L. Rodriguez, unpublished data). If females did not mate within the 3-hr behavioral trial period, we separated the pair and placed females into a new copulation duration trial on a subsequent day. We assigned these females their original randomized treatment but provided them with a different male. In most cases, we gave females up to four chances to copulate: 65% of females mated with the first male, 18% with the second male, 9% with the third male, and 6% with the fourth male. There were two exceptions where we gave females more chances: one female was given five chances (10-min treatment) and one female was given six chances (45-min treatment). If females never mated, we excluded them from this experiment. Within the uninterrupted treatment, there was one mating pair that mated for 9 min. Since copulation duration in the uninterrupted treatment lasted between 75 and 138 min and averaged 109.4 min long, this pair was an outlier. This pair may not have mated but instead may have been oriented in the mating position after an unsuccessful mating attempt (D. Little, personal communication), so we removed the pair from our dataset.

We then tested female receptivity as described above 1 day (both 2021 and 2022 females) and 1 week (2022 females only) after copulation. We also tracked the presence and number of egg masses and offspring females produced (both 2021 and 2022 females) (Figure 1B and C) as well as adult survival (2022 females only).

Statistical analyses

Our first goal was to examine whether our experimental manipulation of copulation duration affected the likelihood of three female reproductive events. We constructed three separate generalized linear mixed models (GLMMs) with a binomial distribution and logit link function. For each model, the response variable was either (1) female receptivity (Y/N), (2) egg mass production (Y/N), or (3) offspring production (Y/N). All GLMMs included mating duration treatment (10 min, 30 min, 45 min, 60 min, and uninterrupted) as an explanatory variable. Site (five different sites), year (2021 and 2022), and mating partner ID were included as random terms. Because we tested female receptivity twice after copulation, we also included time since copulation (1 day or 1 week) as an explanatory variable and female ID as a random term in the receptivity model. We originally included the temperature (°C) at the time of testing receptivity in this model. Temperature was never significant ($p > 0.9$) and did not qualitatively change the results when we removed it, so we kept it out of the model. Sample sizes between these analyses differ due to data collection additions from 2021 to 2022, females not laying any eggs, females not producing any offspring, females escaping, female mortality, host plant mortality, or females being overlooked for testing (Table 2). Sample sizes were smaller for the offspring production (Y/N) model compared to other models because if females did not produce at least one egg mass, they were removed from this analysis.

Our next goal was to examine the total number of offspring produced by females engaged in varying lengths of copulation. We examined the total number of offspring produced by all females in all five mating duration treatments. Thus, all females from each treatment were included in this analysis. We also analyzed the data in the two longer copulation duration treatments (i.e., 60 min and uninterrupted) since

Table 2. Sample sizes for all reproductive events examined in the experiment testing the effect of copulation interruption in *Enchenopa* treehoppers.

Copulation duration	Receptivity (24 hr)	Receptivity (1 week)	Egg production (Y/N)	Offspring production (Y/N)	Total offspring number
10 min	20	12	18	7	18
30 min	20	12	20	9	19
45 min	17	14	18	10	18
60 min	19	13	18	14	16
Uninterrupted	20	14	20	17	19

they produced the most offspring while only three females produced offspring in the shorter copulation duration treatments (i.e., 10-, 30-, and 45-min groups). We constructed two GLMMs with a Poisson distribution (log link function): one for the full dataset and the other composed of a subset of the two longest copulation duration treatments. The number of offspring produced was our response variable. Copulation duration treatment was our explanatory variable and site, year, and mating partner ID were included as random terms.

Our next goal was to investigate whether copulation duration affected adult survival. We first examined if the number of adult days alive after copulation was correlated with the number of offspring that females produced. We constructed two GLMMs with Poisson distributions and log link functions. The number of offspring produced was the response variable and the number of adult days alive after copulation was the explanatory variable. We first included all of the treatments in this analysis. Then, we examined the same correlation with the uninterrupted group only. We included site and mating partner ID as random terms in both models. Finally, we constructed a mixed-effects cox survival model using the "efron" method for handling exact ties in the survival time (*coxme* function and package). We included the number of adult days alive after copulation as the response variable and copulation duration treatment as the explanatory variable. We also included site and mating partner ID as random terms in this model. Year was not included as a random term in either of these models because female survival was not tracked in 2021.

Finally, we analyzed the relationship between number of offspring produced by females that engaged in copulations of different durations in the uninterrupted treatment. We constructed four separate Spearman's rho correlation tests since our data were not distributed normally. We examined the correlations between (1) offspring and copulation duration, (2) egg masses and copulation duration, (3) offspring per egg mass and copulation duration, and (4) offspring and egg masses. We calculated total offspring per egg mass by dividing the total offspring by the total egg masses that females produced. We did this to partition the variation in offspring produced so that we could examine whether copulation duration increases sperm transfer (Arnold and Wade, 1984). All GLMMs were constructed using the *glmmTMB* package (Bolker 2019). All analyses were constructed in R version 4.3.0 (R Core Development Team, 2023).

Results

We found that females that engaged in shorter copulations were more likely to be receptive post-copulation than females engaged in longer copulations (GLMM: $\chi^2 = 19.03$, df = 4, $p \leq 0.001$; Figure 2). This pattern in female receptivity did not change between one day and one week after copulation ($\chi^2 = 2.54$, df = 1, $p = 0.11$; Figure 2). Females that had shorter copulations were also less likely to produce egg masses when compared to females that copulated for longer (GLMM: $\chi^2 = 15.02$, df = 4, $p = 0.004$; Figure 2). Of those females that produced at least one egg mass, copulation duration treatment did not affect the likelihood that females would produce offspring (GLMM: $\chi^2 = 5.35$, df = 4, $p = 0.25$; Figure 2). However, when we compared total offspring number for all females in the experiment (including females that did not produce any egg masses), we found that females that experienced shorter copulation durations produced fewer offspring than females that engaged in longer copulations (GLMM: $\chi^2 = 44.01$, df = 4, $p < 0.001$; Figure 3). Since females in the two longest copulation duration treatments produced the most offspring, we analyzed the number of offspring produced in only these two treatments. We found that there were no differences in offspring production between the 60-min and the uninterrupted groups (GLMM: $\chi^2 = 0.32$, df = 1, $p = 0.57$; Figure 3). This data also strongly suggests that our method for separating the mating pairs did not negatively affect reproduction.

We also found that females that survived longer produced more offspring, including all females in all treatments (GLMM: $\chi^2 = 17.46$, df = 1, $p < 0.001$; Figure 4A), and including only females from the uninterrupted treatment (GLMM: $\chi^2 = 10.13$,

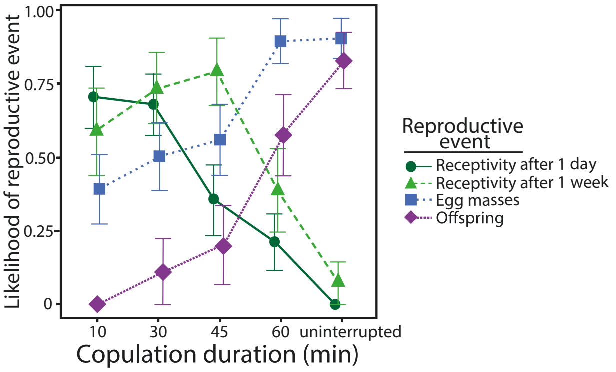

Figure 2. The likelihood of a reproductive event occurring in the experiment testing the effect of copulation interruption in *Enchenopa* treehoppers. Females engaged in shorter copulations are more likely to be receptive after copulating (circles and triangles and solid and long dashed lines) and less likely to produce egg masses (squares and short dashed line) and offspring (diamonds and dotted line) than females engaged in longer copulations. Sample sizes are lower in the offspring production analysis because females must have laid at least one egg mass.

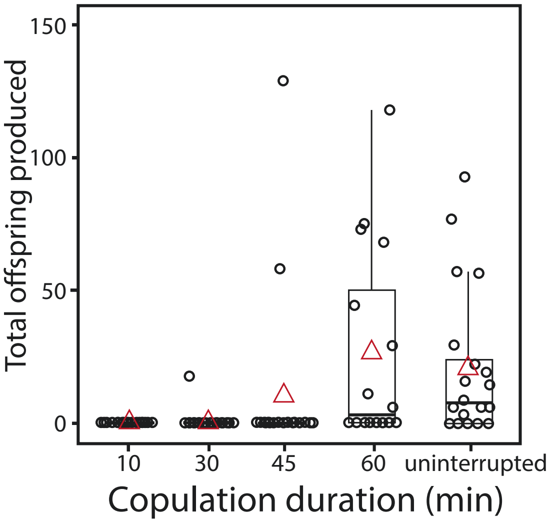

Figure 3. *Enchenopa* females produced more offspring when they engaged in longer copulations in the experiment testing the effect of copulation interruption. There was, however, no difference in the number of offspring produced between the two longest copulation times (i.e., 60 min and uninterrupted).

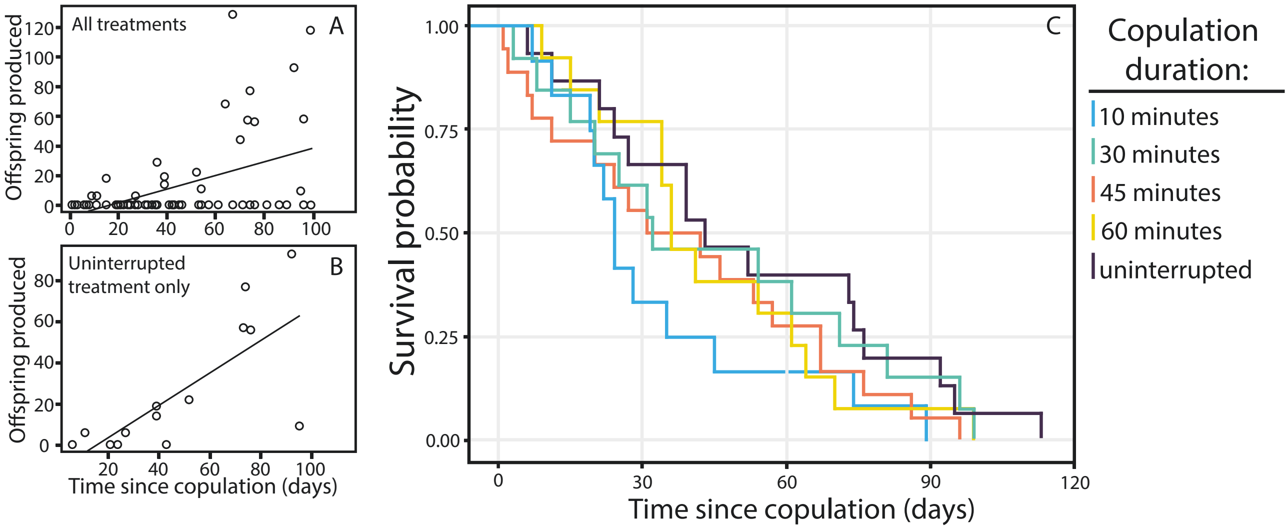

Figure 4. The longer *Enchenopa* females survived after copulation, the more offspring they produced in all treatments (A) and in the uninterrupted treatment (B). However, copulation duration did not affect adult female survival after copulation (C).

df = 1, $p = 0.002$; Figure 4B). However, copulation duration did not affect the number of days a female survived as an adult after copulation (mixed-effects cox model: $\chi^2 = 5.83$, df = 4, $p = 0.21$; Figure 4C).

Finally, we analyzed female reproduction in the uninterrupted treatment only. We found that pairs assigned to the uninterrupted treatment copulated for 109.4 ± 3.13 *SE* minutes, on average. Copulations in this treatment lasted between 75 and 138 min and this variation in duration may be due to body size, although we did not measure body size in this study (Parker & Simmons, 1994; Parker et al., 1999). Mating pairs appeared to end copulation gently and cooperatively without either sex wrenching, kicking, or squirming to get out of the pair formation. Most times, males and females remained next to or near each other post-copulation. Copulation duration in the uninterrupted treatment was not correlated with the number of offspring (rho = −0.08, $p = 0.74$), the number of egg masses (rho = −0.28, $p = 0.24$), nor the offspring per egg mass they produced (rho = 0.39, $p = 0.14$; Figure 5). The number of egg masses produced by females in the uninterrupted treatment was correlated with the number of offspring produced (rho = 0.9, $p < 0.001$; Figure 5). Five females did not produce any offspring in the uninterrupted treatment (Table 3). Of these females, only one female was receptive again after one week (Table 3, Figure 5) and two females survived less than 7 days post-copulation (Table 3).

Discussion

We tested a suite of potentially complementary hypotheses that seek to explain prolonged copulations in cases of low female remating rates. We worked with *Enchenopa* treehoppers, which have a very low female remating rate and can copulate for several hours (Leith et al., 2020; Sullivan-Beckers & Cocroft, 2010). Following this prolonged copulation, females drastically reduce their receptivity (Cocroft et al., 2008; Wood, 1993).

We experimentally manipulated copulation length and found that when *Enchenopa* pairs mate for prolonged periods of time, female receptivity becomes extremely low and offspring production is high when compared to pairs that mated for shorter time periods. We found that offspring number did not differ when we compared the two highest copulation duration treatments; however, female receptivity tended to continue to decrease beyond copulations of 60 min and was lowest (albeit not significantly so in post-hoc comparisons) in the uninterrupted treatment.

These results provide support for both the sperm transfer and receptivity regulation hypotheses (Table 1). *Enchenopa* treehoppers may engage in prolonged copulations because the transfer of sperm and putative accessory substances takes at least ca. 60 min or more. Interestingly, sperm transfer seems to have peaked before accessory substances that may help lower female receptivity, although receptivity does not differ statistically between the 60-min and uninterrupted groups. Females may take a cue from the event of copulation to begin egg production or egg laying, although we find females that produced egg masses in all experimental groups. We consider it unlikely, however, that such a cue would take such a long copulation time to take effect (e.g., beyond 45 min) while still not being long enough for much sperm transfer.

Mating beyond 60-min does not generate more offspring but does appear to regulate female receptivity. This result suggests that males may transfer accessory substances to females that females may use in regulating their receptivity. Males in this species likely have accessory glands that may produce receptivity reducing substances since they are present in developing Membracidae homopterans (Singh & Mishra, 1973), adult male Homoptera (Tsai & Perrier, 1993), and a related treehopper *Ennya* sp. (Membracidae) (Ximena Miranda, personal communication). We note that we have only provided indirect evidence that *Enchenopa* males may transfer receptivity reducing accessory substances. Future investigations should examine whether males produce and transfer these substances in the ejaculate to females and examine their direct effect.

The value for males of attempting to induce a lack of receptivity in females is highlighted by the strength of pre-copulatory female mate choice in *Enchenopa*. Males use competitive signaling to court females and females are extremely selective in choosing mates (Cirino et al., 2023; Cocroft et al., 2008; Rodríguez et al. 2004b, 2006, 2013; Speck 2022),

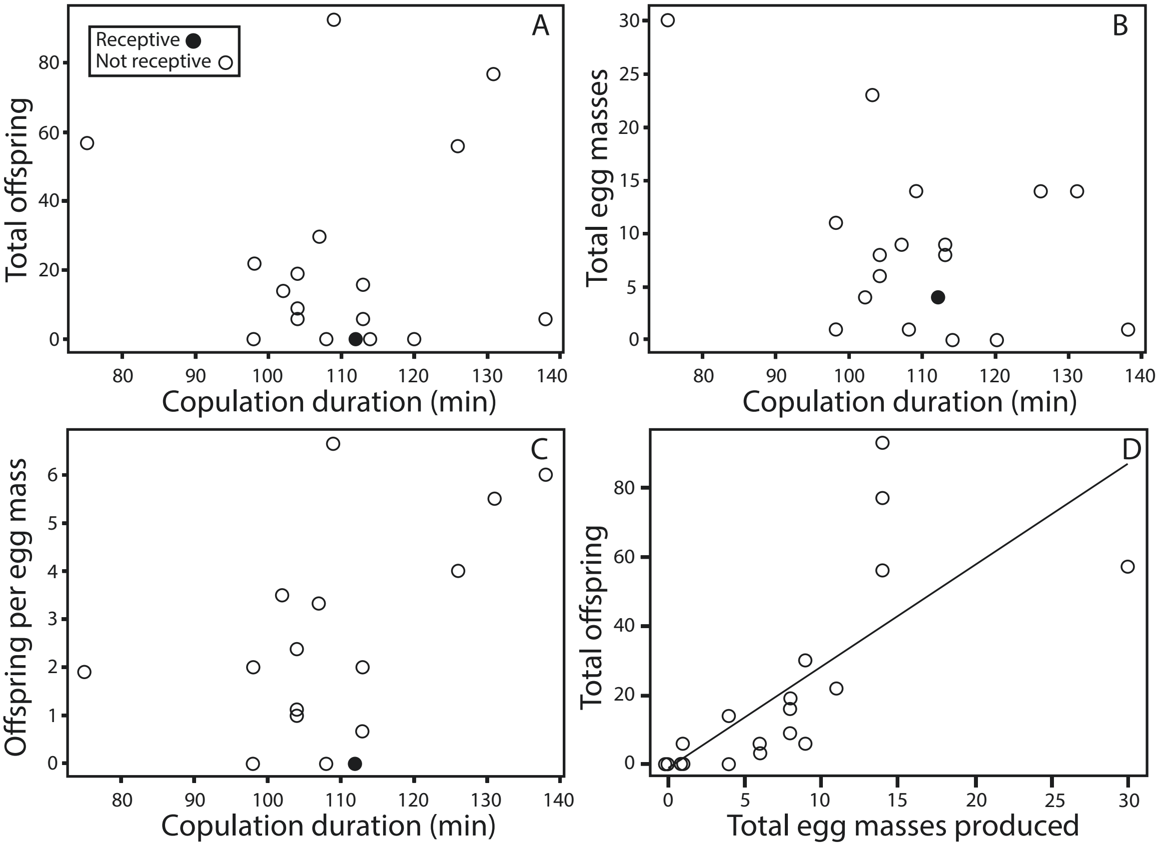

Figure 5. Reproductive output by *Enchenopa* females in the uninterrupted treatment. Each female is represented by a circle. Only one female that did not produce any offspring was receptive 1 week post-copulation (closed circle). We did not detect significant correlations between (A) copulation duration and total offspring, (B) copulation duration and total egg masses, or (C) copulation duration and offspring per egg mass in this treatment. The sample size of graph A differs from graph D because we did not record the copulation duration of one mating pair from 2021. The sample size of graph C is different from graphs A and B because females had to produce at least one egg mass to be included in this correlation. (D) The amount of egg masses is correlated with the number of offspring they produced. Two females did not produce any egg masses or offspring and two other females produced one egg mass and no offspring (one of these females was receptive after copulation). Thus, the points for these females are open and jittered on this graph for visualization purposes.

Table 3. Survival and reproduction data for the five females that did not produce any offspring from the uninterrupted treatment. All females except one female from 2021, indicated in parentheses, were from the 2022 *E. binotata* population. Although survivorship was not tracked for females in 2021, female 60 did not survive to one week post-copulation. Thus, we could not assess her receptivity at the 1-week timepoint.

Female ID	Copulation duration (minutes)	Survival post-copulation (days)	Egg masses produced	Offspring produced	Receptive after 1 week
25	108	21	1	0	no
59	112	43	4	0	yes
70	114	24	0	0	no
152	98	6	1	0	no
60 (2021)	120	N/A	0	0	N/A

Note. N/A = not available.

which manifested in only 54.4% of our behavior trials ending in copulation. Indeed, most males fail to mate with only ca. 20% securing one copulation and fewer obtaining more (Sullivan-Beckers & Cocroft, 2010). Thus, with strong female mate choice combined with high male mortality during the breeding season (Supplementary Figure 1), it seems that males would benefit from prolonging a copulation if it helped lower the risk of post-copulatory competition.

Note that we do not mean to say that males rather than females control copulation duration. Females may sense that they have received enough sperm to fertilize all of their eggs and thus do not need to re-mate to acquire more. Indeed, the females in this study that laid egg masses that did not hatch appeared to be more receptive after copulation than those that had eggs that hatched—indicating a lack of sperm (Supplementary Table 1). When we examined the uninterrupted treatment alone, we found a trend in which offspring per egg mass increased with copulation duration, although this was not a significant correlation. However, we also found that some females that mated for prolonged periods did not have any or enough sperm to fertilize their eggs, yet they were still not receptive after copulation (2/17 or 12%). Thus, females might instead use the time spent mating as an indicator of the amount of sperm they have received. The trends described above arise from low sample sizes, though, so future work should investigate these patterns.

Our results also provide some evidence against the nutritional gift hypothesis. Although females that copulated longer produced more offspring, which would be consistent with the nutritional gift hypothesis, they did not live longer, which was expected under this hypothesis. Additionally, the number of egg masses produced was not positively correlated with copulation duration in the uninterrupted treatment providing more evidence against this hypothesis. Our results also provide strong evidence against the life history manipulation hypothesis as females that copulated longer did not experience decreased mortality as expected. However, we note that we did not check for changes in their reproductive schedule (i.e., timing of oviposition).

It is surprising that ejaculate transfer is protracted in *Enchenopa* treehoppers (ca. 60 min). One explanation for this might be their genital morphology. Long male genitalia may need time to navigate the reproductive tract of females before they can inseminate and produce offspring as lygaeid seed bugs require (Greenway & Shuker, 2015; Micholitsch et al., 2000). Similarly, tortoise beetles that have longer genitalic flagellum mate for longer and have greater paternity than those with shorter genitalia that mate for shorter time periods (Rodríguez et al. 2004b). Bushcrickets that have genital structures that function in courtship have longer mating times than those that do not have these structures (Vahed et al., 2011). However, *Enchenopa* male genital morphology (i.e., aedeagus and styles) does not seem to influence sperm transfer (Macchiano, 2023) and it does not appear that *Enchenopa* males have any genital structures used for courtship that would prolong copulations (Pratt & Wood, 1993; Rodríguez & Al-Wathiqui, 2011). Future work is needed to explain why sperm transfer should take so long in this species.

One intriguing possibility suggested by our data is that the evolution of prolonged copulations in species that have a low female remating rate may be related to post-copulatory sexual selection (Dougherty et al., 2016). Consider a case in which copulation length is related to the transfer of accessory substances that help decrease subsequent female receptivity (Aisenberg & Costa, 2005; Gillott, 2003; Leopold et al., 1971; Murvosh et al., 1964; Simmons & Siva-Jothy, 1998; Yamane, 2013; Yamane et al., 2008). If females evolve to accept such a chemical inducement, males might in effect be able to prevent further post-copulatory competition by lowering female remating rates (Parker, 1970). Thus, the feature of the mating system where most females mate once that seems to preclude post-copulatory competition would in fact be explained by it. Research on feedbacks between pre- and post-copulatory sexual selection may be highly illuminative for understanding the evolution of mating systems.

Supplementary material

Supplementary material is available at *Journal of Evolutionary Biology* online.

Data availability

The data underlying this paper are available in the Dryad repository at https://datadryad.org/stash/share/cqlIuxxWP-6JukLMFCAxK93ZKunswsLmp_vquvhwSV4U.

Author contributions

Lauren A. Cirino (Conceptualization [lead], Data curation [lead], Formal analysis [lead], Investigation [lead], Methodology [lead], Project administration [lead], Supervision [equal], Validation [equal], Visualization [lead], Writing—original draft [lead], Writing—review & editing [lead]), Ariel Noriega Rodriguez (Data curation [equal], Investigation [equal], Methodology [equal], Visualization [equal], Writing—review & editing [equal]), Sage A. DeLong (Data curation [equal], Investigation [equal], Methodology [equal], Writing—review & editing [equal]), and Rafael L. Rodriguez (Conceptualization [supporting], Formal analysis [supporting], Funding acquisition [lead], Investigation [supporting], Methodology [supporting], Project administration [equal], Resources [lead], Supervision [equal], Validation [equal], Visualization [supporting], Writing—original draft [equal], Writing—review & editing [equal])

Funding

This research was supported by the U.S. National Science Foundation Grant IOS – 1855962 to R.L.R. and C. Desjonquères. A.N.R. and S.A.D. were also supported by UWM Support for Undergraduate Research Fellows (SURF) awards.

Acknowledgments

We thank Sara Seidita and Ian Gallagher for their help with insect rearing and animal and plant care as well as Chance LaCombe for help with the 2021 behavior trials. We would also like to thank Paul Engevold for his help and support at the UWM Greenhouse.

Conflicts of interest

None declared

References

Aisenberg, A., & Costa, F. G. (2005). Females mated without sperm transfer maintain high sexual receptivity in the wolf spider *Schizocosa malitiosa*. *Ethology*, *111*(6), 545–558. https://doi.org/10.1111/j.1439-0310.2005.01077.x

Alcock, J. (1994). Post-insemination associations between males and females in insects—The mate-guarding hypothesis. *Annual Review of Entomology*, *39*(1), 1–21. https://doi.org/10.1146/annurev.ento.39.1.1

Amin, M. R., Bussiere, L. F., & Goulson, D. (2012). Effects of male age and size on mating success in the bumblebee *Bombus terrestris*. *Journal of Insect Behavior*, *25*(4), 362–374. https://doi.org/10.1007/s10905-011-9306-4

Andersson, M. B. (1994). *Sexual selection*. Princeton University Press.

Arnold, S. J., & Wade, M. J. (1984). On the measurement of natural and sexual selection: Applications. *Evolution*, *38*(4), 720–734. https://doi.org/10.1111/j.1558-5646.1984.tb00345.x

Arnqvist, G., & Nilsson, T. (2000). The evolution of polyandry: Multiple mating and female fitness in insects. *Animal Behaviour*, *60*(2), 145–164. https://doi.org/10.1006/anbe.2000.1446

Bateman, A. J. (1948). Intra-sexual selection in *Drosophila*. *Heredity*, *2*(Pt. 3), 349–368. https://doi.org/10.1038/hdy.1948.21

Boggs, C. L., & Gilbert, L. E. (1979). Male contribution to egg production in butterflies: Evidence for transfer of nutrients at mating. *Science*, *206*(4414), 83–84. https://doi.org/10.1126/science.206.4414.83

Bolker, B. (2019). Getting started with glmmTMB package. Cran. *R-project vingette*, *9*.

Brennan, P. L. R., & Orbach, D. N. (2020). Copulatory behavior and its relationship to genital morphology. *Advances in the Study of Behavior*, *52*, 65–122. https://doi.org/10.1016/bs.asb.2020.01.001

Bretman, A., Fricke, C., Hetherington, P., … Chapman, T. (2010). Exposure to rivals and plastic responses to sperm competition in *Drosophila melanogaster*. *Behavioral Ecology*, *21*(2), 317–321. https://doi.org/10.1093/beheco/arp189

Brown, M. J. F., & Baer, B. (2005). The evolutionary significance of long copulation duration in bumble bees. *Apidologie*, *36*(2), 157–167. https://doi.org/10.1051/apido:2005008

Campbell, V., & Fairbairn, D. J. (2001). Prolonged copulation and the internal dynamics of sperm transfer in the water strider *Aquarius remigis*. *Canadian Journal of Zoology*, *79*(10), 1801–1812. https://doi.org/10.1139/z01-148

Carroll, S. P. (1991). The adaptive significance of mate guarding in the soapberry bug, *Jadera haematoloma* (Hemiptera, Rhopalidae). *Journal of Insect Behavior*, *4*(4), 509–530. https://doi.org/10.1007/bf01049335

Chapman, T., Liddle, L. F., Kalb, J. M., … Partridge, L. (1995). Cost of mating in *Drosophila melanogaster* females is mediated by accessory gland products. *Nature*, *373*(6511), 241–244. https://doi.org/10.1038/373241a0

Cirino, L. A., Gallagher, I. D., Desjonquères, C., & Rodrìguez, R. L. (2023). Robust mate preferences despite means and opportunity for mate choice copying in an insect. *Animal Behaviour*, *200*, 137–146. https://doi.org/10.1016/j.anbehav.2023.03.018

Cocroft, R. B., & Rodriguez, R. L. (2005). The behavioral ecology of insect vibrational communication. *Bioscience*, *55*(4), 323–334. https://doi.org/10.1641/0006-3568(2005)055[0323:tbeoiv]2.0.co;2

Cocroft, R. B., Rodriguez, R. L., & Hunt, R. E. (2008). Host shifts, the evolution of communication, and speciation in the *Enchenopa binotata* species complex of treehoppers. In K. Tilmon (Ed.), *Specialization, speciation, and radiation: The evolutionary biology of herbivorous insects* (pp. 88–100). University of California Press.

Cocroft, R. B., Rodriguez, R. L., & Hunt, R. E. (2010). Host shifts and signal divergence: Mating signals covary with host use in a complex of specialized plant-feeding insects. *Biological Journal of the Linnean Society*, *99*(1), 60–72.

Darwin, C. (1871). *Descent of man and selection in relation to sex*. D. Appleton and Company.

Dougherty, L. R., Simmons, L. W., & Shuker, D. M. (2016). Postcopulatory sexual selection when a female mates once. *Animal Behaviour*, *116*, 13–16. https://doi.org/10.1016/j.anbehav.2016.03.003

Eberhard, W. G. (1996). *Female control: Sexual selection by cryptic female choice*. Princeton University Press.

Eberhard, W. G. (2009). Postcopulatory sexual selection: Darwin's omission and its consequences. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(Suppl. 1), 10025–10032. https://doi.org/10.1073/pnas.0901217106

Fowler-Finn, K. D., Kilmer, J. T., Cruz, D. C., & Rodriguez, R. L. (2018). Female mate choice of male signals is unlikely to promote ecological adaptation in *Enchenopa* treehoppers (Hemiptera: Membracidae). *Ecology and Evolution*, *8*(4), 2146–2159. https://doi.org/10.1002/ece3.3817

Fowler-Finn, K. D., & Rodríguez, R. L. (2013). Repeatability of mate preference functions in *Enchenopa* treehoppers (Hemiptera: Membracidae). *Animal Behaviour*, *85*, 493–499. https://doi.org/10.1016/j.anbehav.2012.12.015

Friesen, C. R., Uhrig, E. J., Bentz, E. J., … Mason, R. T. (2017). Correlated evolution of sexually selected traits: Interspecific variation in ejaculates, sperm morphology, copulatory mate guarding, and body size in two sympatric species of garter snakes. *Behavioral Ecology and Sociobiology*, *71*(12), Article 180. https://doi.org/10.1007/s00265-017-2414-1

Friesen, C. R., Uhrig, E. J., Mason, R. T., & Brennan, P. L. R. (2016). Female behaviour and the interaction of male and female genital traits mediate sperm transfer during mating. *Journal of Evolutionary Biology*, *29*(5), 952–964. https://doi.org/10.1111/jeb.12836

Fromonteil, S., Marie-Orleach, L., Winkler, L., & Janicke, T. (2023). Sexual selection in females and the evolution of polyandry. *PLoS Biology*, *21*(1), e3001916. https://doi.org/10.1371/journal.pbio.3001916

Garcia-Gonzalez, F., & Gomendio, M. (2004). Adjustment of copula duration and ejaculate size according to the risk of sperm competition in the golden egg bug (*Phyllomorpha laciniata*). *Behavioral Ecology*, *15*(1), 23–30. https://doi.org/10.1093/beheco/arg095

Gillott, C. (2003). Male accessory gland secretions: Modulators of female reproductive physiology and behavior. *Annual Review of Entomology*, *48*, 163–184. https://doi.org/10.1146/annurev.ento.48.091801.112657

Greenway, E. G., Cirino, L. A., Wilner, D., … Miller, C. W. (2020). Extreme variation in testes size in an insect is linked to recent mating activity. *Journal of Evolutionary Biology*, *33*, 142–150.

Greenway, E. V., & Shuker, D. M. (2015). The repeatability of mating failure in a polyandrous bug. *Journal of Evolutionary Biology*, *28*(8), 1578–1582. https://doi.org/10.1111/jeb.12678

Hamilton, K. G. A., & Cocroft, R. B. (2009). Establishing the identity of existing names in the North American *Enchenopa binotata* species complex of treehoppers (Hemiptera: Membracidae). *Entomological News*, *120*(5), 554–565. https://doi.org/10.3157/021.120.0513

Hill, P. S. M. (2008). *Vibrational communication in animals*. Harvard University Press.

Hughes, L., Chang, B. S. W., Wagner, D., & Pierce, N. E. (2000). Effects of mating history on ejaculate size, fecundity, longevity, and copulation duration in the ant-tended lycaenid butterfly, *Jalmenus evagoras*. *Behavioral Ecology and Sociobiology*, *47*(3), 119–128. https://doi.org/10.1007/s002650050002

Janicke, T., Haderer, I. K., Lajeunesse, M. J., & Anthes, N. (2016). Darwinian sex roles confirmed across the animal kingdom. *Science Advances*, *2*(2), e1500983. https://doi.org/10.1126/sciadv.1500983

Kvarnemo, C., & Simmons, L. W. (2013). Polyandry as a mediator of sexual selection before and after mating. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *368*(1613), 20120042. https://doi.org/10.1098/rstb.2012.0042

Leith, N. T., Jocson, D. I., & Fowler-Finn, K. D. (2020). Temperature-related breakdowns in the coordination of mating in *Enchenopa binotata* treehoppers (Hemiptera: Membracidae). *Ethology*, *126*(9), 870–882. https://doi.org/10.1111/eth.13033

Leopold, R. A., Terranova, A. C., & Swilley, E. M. (1971). Mating refusal in *Musca domestica*: Effects of repeated mating and decerebration upon frequency and duration of copulation. *The Journal of Experimental Zoology*, *176*(3), 353–359. https://doi.org/10.1002/jez.1401760310

Linn, C. D., Molina, Y., Difatta, J., & Christenson, T. E. (2007). The adaptive advantage of prolonged mating: A test of alternative hypotheses. *Animal Behaviour*, *74*(3), 481–485. https://doi.org/10.1016/j.anbehav.2007.02.004

Ludwig, A. N., & Walsh, P. J. (2004). Sperm storage and use in laboratory-reared *Aplysia californica*, a simultaneous hermaphrodite. *Marine Biology*, *145*(4), 727–732. https://doi.org/10.1007/s00227-004-1358-y

Macchiano, A. (2023). *Ambient and developmental temperature affects the expression of reproductive traits and mating success in variable thermal environments in Enchenopa binotata treehoppers* [*Doctoral dissertation*, Saint Louis University], ProQuest Dissertations Publishing. 30489172.

Micholitsch, T., Krügel, P., & Pass, G. (2000). Insemination and fertilization in the seed bug *Lygaeus simulans* (Heteroptera: Lygaeidae). *European Journal of Entomology*, *97*(1), 13–18. https://doi.org/10.14411/eje.2000.003

Murvosh, C. M., Fye, R. L., & LaBrecque, G. C. (1964). Studies on the mating behavior of the house fly, *Musca domestica* L. *The Ohio Journal of Science*, *64*, 264–271.

Orr, A. G., & Rutowski, R. L. (1991). The function of the sphragis in *Cressida cressida* (Fab) (Lepidoptera, Papilionidae)—A visual deterrent to copulation attempts. *Journal of Natural History*, *25*(3), 703–710. https://doi.org/10.1080/00222939100770461

Parker, G. A. (1970). Sperm competition and its evolutionary consequences in insects. *Biological Reviews*, *45*(4), 525–567. https://doi.org/10.1111/j.1469-185x.1970.tb01176.x

Parker, G. A., & Simmons, L. W. (1994). Evolution of phenotypic optima and copula duration in dungflies. *Nature*, *370*(6484), 53–56. https://doi.org/10.1038/370053a0

Parker, G. A., Simmons, L. W., Stockley, P., … Charnov, E. L. (1999). Optimal copula duration in yellow dung flies: Effects of female size and egg content. *Animal Behaviour*, *57*(4), 795–805. https://doi.org/10.1006/anbe.1998.1034

Pratt, G., & Wood, T. K. (1993). Genitalic analysis of males and females in the *Enchenopa binotata* (Say) complex (Membracidae: Homoptera). *Proceedings of the Entomological Society of Washington*, *95*(4), 574–582.

R Core Development Team. (2023). *R: A language and environment for statistical computing*. R Foundation for Statistical Computing. https://www.r-project.org/

Rodríguez, R. L., & Al-Wathiqui, N. (2011). Genotype × environment interaction is weaker in genitalia than in mating signals and body traits in *Enchenopa* treehoppers (Hemiptera: Membracidae). *Genetica*, *139*(7), 871–884. https://doi.org/10.1007/s10709-011-9591-z

Rodríguez, R. L., & Cocroft, R. B. (2006). Divergence in female duetting signals in the *Enchenopa binotata* species complex of treehoppers (Hemiptera: Membracidae). *Ethology*, *112*(12), 1231–1238. https://doi.org/10.1111/j.1439-0310.2006.01285.x

Rodríguez, R. L., Haen, C., Cocroft, R. B., & Fowler-Finn, K. D. (2012). Males adjust signaling effort based on female mate-preference cues. *Behavioral Ecology*, *23*(6), 1218–1225. https://doi.org/10.1093/beheco/ars105

Rodríguez, R. L., Hallett, J., Kilmer, T., & Fowler-Finn, K. (2013). Curves as traits: Genetic and environmental variation in mate preference functions. *Journal of Evolutionary Biology*, *26*, 434–442. https://doi.org/10.1111/jeb.12061

Rodríguez, R. L., Ramaswamy, K., & Cocroft, R. B. (2006). Evidence that female preferences have shaped male signal evolution in a clade of specialized plant-feeding insects. *Proceedings Biological Sciences*, *273*(1601), 2585–2593. https://doi.org/10.1098/rspb.2006.3635

Rodríguez, R. L., Sullivan, L. E., & Cocroft, R. B. (2004a). Vibrational communication and reproductive isolation in the *Enchenopa binotata* species complex of treehoppers (Hemiptera: Membracidae). *Evolution*, *58*(3), 571–578. https://doi.org/10.1111/j.0014-3820.2004.tb01679.x

Rodríguez, R. L., Wojcinski, J. E., & Maliszewski, J. (2018). Between-group variation in *Enchenopa* treehopper juvenile signaling (Hemiptera: Membracidae). *Ethology Ecology & Evolution*, *30*(3), 245–255. https://doi.org/10.1080/03949370.2017.1347585

Rodríguez, V., Windsor, D. M., & Eberhard, W. G. (2004b). Tortoise beetle genitalia and demonstrations of a sexually selected advantage for flagellum length in *Chelymorpha alternans* (Chrysomelidae, Cassidini, Stolaini). In P. Jolivet, J. A. Santiago-Blay, & M. Schmitt (Eds.), *New developments in the biology of Chrysomelidae* (pp. 739–748). Brill. https://doi.org/10.1163/9789004475335_063

Schneider, J. M., Gilberg, S., Fromhage, L., & Uhl, G. (2006). Sexual conflict over copulation duration in a cannibalistic spider. *Animal Behaviour*, *71*(4), 781–788. https://doi.org/10.1016/j.anbehav.2005.05.012

Simmons, L. W., & Siva-Jothy, M. T. (1998). Sperm competition in insects: Mechanisms and the potential for selection. In T. R. Birkhead, & A. P. Møller (Eds.), *Sperm competition and sexual selection* (pp. 341–414). Academic Press Ltd.

Singh, M. P., & Mishra, S. K. (1973). Studies on the development of male reproductive organs of *Atinotus elongatus* dist. (Membracidae, Homoptera). *Kontyû*, *41*(2), 228–233.

Sirot, L. K., Buehner, N. A., Fiumera, A. C., & Wolfner, M. F. (2009). Seminal fluid protein depletion and replenishment in the fruit fly, *Drosophila melanogaster*: An ELISA-based method for tracking individual ejaculates. *Behavioral Ecology and Sociobiology*, *63*(10), 1505–1513. https://doi.org/10.1007/s00265-009-0806-6

Speck, B. L. V. 2022. *Architecture of mate choice decisions in Enchenopa treehoppers* [*Doctoral dissertation*, University of Wisconsin-Milwaukee]. ProQuest Dissertations Publishing. 29210649.

Sullivan-Beckers, L., & Cocroft, R. B. (2010). The importance of female choice, male–male competition, and signal transmission as causes of selection on mating signals. *Evolution*, *64*(11), 3158–3171. https://doi.org/10.1111/j.1558-5646.2010.01073.x

Sultana, A. (2022). Effects of mating on the copulation duration, fecundity and fertility of *Aedes albopictus* and *Aedes flavopictus*. *Journal of Experimental Biosciences*, *13*(2), 1–10.

Tallamy, D. W., & Wood, T. K. (1986). Convergence patterns in subsocial insects. *Annual Review of Entomology*, *31*, 369–390. https://doi.org/10.1146/annurev.en.31.010186.002101

Thornhill, R. (1983). Cryptic female choice and its implications in the scorpionfly *Harpobittacus nigriceps*. *American Naturalist*, *122*(6), 765–788. https://doi.org/10.1086/284170

Tonnabel, J., David, P., & Pannell, J. R. (2019). Do metrics of sexual selection conform to Bateman's principles in a wind-pollinated plant? *Proceedings of the Royal Society B: Biological Sciences*, *286*(1905), 20190532. https://doi.org/10.1098/rspb.2019.0532

Tsai, J. H., & Perrier, J. L. (1993). Morphology of the digestive and reproductive systems of *Peregrinus maidis* (Homoptera, Delphacidae). *Florida Entomologist*, *76*(3), 428–436. https://doi.org/10.2307/3495643

Urquizo, O. N., Veliz, D., Torrico-Bazoberry, D., … Pinto, C. F. (2020). Reproductive and brood-rearing strategies in *Alchisme grossa* (Hemiptera: Membracidae): Genetic analyses of kinship relationships. *Insectes Sociaux*, *67*(3), 347–354. https://doi.org/10.1007/s00040-020-00776-3

Vahed, K., Lehmann, A. W., Gilbert, J. D., & Lehmann, G. U. C. (2011). Increased copulation duration before ejaculate transfer is associated with larger spermatophores, and male genital titillators, across bushcricket taxa. *Journal of Evolutionary Biology*, *24*(9), 1960–1968. https://doi.org/10.1111/j.1420-9101.2011.02325.x

Walker, S. E., & Ford, N. B. (1996). Courtship and mating behavior in the brown house snake *Lamprophis fulginosus*. *Journal of Herpetology*, *30*(3), 416–418. https://doi.org/10.2307/1565183

Wiklund, C., Kaitala, A., Lindfors, V., & Abenius, J. (1993). Polyandry and its effects on female reproduction in the green-veined white butterfly (*Pieris napi* L.). *Behavioral Ecology and Sociobiology*, *33*(1), 25–33. https://doi.org/10.1007/bf00164343

Wood, T. K. (1974). Aggregating behavior of *Umbonia crassicornis* (Homoptera: Membracidae). *Canadian Entomologist*, *106*(2), 169–173. https://doi.org/10.4039/ent106169-2

Wood, T. K. (1993). Speciation of the *Enchenopa binotata* complex (Insecta: Homoptera: Membracidae). In D. R. Less & D. Edwards (Eds.), *Evolutionary patterns and processes* (pp. 299–317). Academic Press.

Wood, T. K., & Guttman, S. I. (1982). Ecological and behavioral basis for reproductive isolation in the sympatric *Enchenopa binotata* complex (Homoptera: Membracidae). *Evolution*, *36*(2), 233–242. https://doi.org/10.1111/j.1558-5646.1982.tb05036.x

Xu, J., & Wang, Q. (2011). Seminal fluid reduces female longevity and stimulates egg production and sperm trigger oviposition in a moth. *Journal of Insect Physiology*, *57*(3), 385–390. https://doi.org/10.1016/j.jinsphys.2010.12.006

Yamane, T. (2013). Reduction of female mating receptivity after injection of male-derived extracts in *Trigonotylus caelestialium* (Heteroptera: Miridae). *Annals of the Entomological Society of America*, *106*(2), 258–264. https://doi.org/10.1603/an12079

Yamane, T., Miyatake, T., & Kimura, Y. (2008). Female mating receptivity after injection of male-derived extracts in *Callosobruchus maculatus*. *Journal of Insect Physiology*, *54*(12), 1522–1527. https://doi.org/10.1016/j.jinsphys.2008.09.001

Zink, A. G. (2003). Quantifying the costs and benefits of parental care in female treehoppers. *Behavioral Ecology*, *14*(5), 687–693. https://doi.org/10.1093/beheco/arg044