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Proximate and Evolutionary Causes of Sexual Size Dimorphism in the Crab Spider *Mecaphesa celer*

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PROXIMATE AND EVOLUTIONARY CAUSES OF SEXUAL SIZE DIMORPHISM IN THE CRAB SPIDER *MECAPHESA CELER*

by

Marie Claire Chelini

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PROXIMATE AND EVOLUTIONARY CAUSES OF SEXUAL SIZE DIMORPHISM IN THE CRAB SPIDER *MECAPHESA CELER*

Marie-Claire Chelini, Ph.D.

University of Nebraska, 2016

Advisor: Eileen A. Hebets

Animal species' body sizes result from the balance between selection for survival and selection for reproduction. In species with sexual size dimorphism (SSD), this balance differs between females and males, resulting in distinct sizes despite similar constraints. I used an integrative approach to understand how sexual section, and differences in developmental trajectories and metabolic physiology, resulted in the female biased SSD of the crab spider *Mecaphesa celer* (Thomisidae). SSD in spiders is often assumed to be a consequence of selection for early male maturation, which should provide males with additional mating opportunities. My results allow us to discard mate choice and differential fitness benefits as sexually selected drivers of *M. celer*'s SSD. Interestingly, I found evidence that *M. celer* females may mate with multiple males, and that, in such instances, eggs are fertilized by a mix of the males' sperm. Such sperm mixing contradicts the hypothesis that *M. celer* males benefit from early-male maturation, as early-matured males do not necessarily fertilize most of a females' eggs. To gain a better understanding of the relation between *M. celer*'s SSD and early male maturation, I identified the proximate mechanisms underlying differences in size between females and males, as well as the effects of the environment on the degree of SSD. Female *M. celer* reach larger sizes by growing faster and for longer than males, although both sexes have the same metabolism at rest. Also, female, but not male size, may be influenced by the interaction between

diet and temperature. Integrating all of these results, I followed the reproductive season of a population of *M. celer* in the wild and found evidences that early maturation does indeed grant males with increased mating opportunities. Interestingly, I also found that the timing of male maturation is not proportional to female and male size, challenging the relationship between early maturation and SSD. My research offers a new perspective to the study of sexual dimorphism evolution, highlighting the importance of studying both sexes from an integrative perspective and shedding light on the developmental processes underlying SSD.

To my parents, who gave me unconditional support to cross the world in search of questions, to crab spiders, that unknowingly give me motivation to answer them, and to my husband, who puts me back on my feet every time I fail to do so.

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OVERVIEW

Selection for survival and selection for reproduction are the main processes leading to differences in female and male body size (Shine 1989; Blanckenhorn 2005). Selection, however, has its limits: the degree to which a group of organisms diverge from its ancestral state depends on its physiological and phylogenetic constraints and can be greatly influenced by its environment (Blanckenhorn 2000). Understanding how and why organisms have a specific size requires that we understand how selection for survival and selection for reproduction interact in each sex, how physiology and phylogeny mediate this interaction, and, finally, how does the environment influence the expression of phenotypic traits.

In species with sexual size dimorphism (SSD), differences in size between the sexes may result from directional selection on one sex's size only or disruptive selection due to different sources, directions, or intensities of selection acting on each sex independently (Fairbairn 2005). Physiological constraints and genetic correlations are expected to maintain the size of both sexes within similar ranges (the null hypothesis of size equality - Hedrick & Temeles 1989; Andersson 1996). Yet, differences in female and male sizes are not only very common but sometimes quite extreme, such as in many examples of female biased SSD (Blanckenhorn 2000; Stillwell *et al.* 2010).


Fig. 1: *Mecaphesa celer* female (bottom) and male

Crab spiders (Thomisidae) are among the most extreme examples of female biased SSD, with females being up to 10 times the size of males and weighing up to 100 times the mass of males (Legrand & Morse 2000; Morse 2007a; Chelini &

Hebets 2016). The evolution of female-biased SSD in spiders is often attributed to early-male maturation and male-male competition for virgin females (protandry), particularly in species where females mate only once and/or have first male sperm priority (Vollrath & Parker 1992; Dodson & Beck 1993a; Elgar 1998; Legrand & Morse 2000; Morse 2013a). The relation between competition for virgin females and SSD has, however, seldom been tested empirically. In the following four studies, I explored the role of selection for reproduction in the evolution of SSD in a species of flower-dwelling crab spider, *Mecaphesa celer* (Fig. 1) and identify physiological differences between the sexes that may constrain or facilitate the expression of disparate sizes (Fig. 2). Using a combination of laboratory mating trials and field observations, I tested if SSD was selected for through precopulatory or postcopulatory benefits. By following the development of laboratory-raised spiderlings, I explored the proximate causes of SSD, and tested the influence of environmental factors on the degree of SSD of this species.

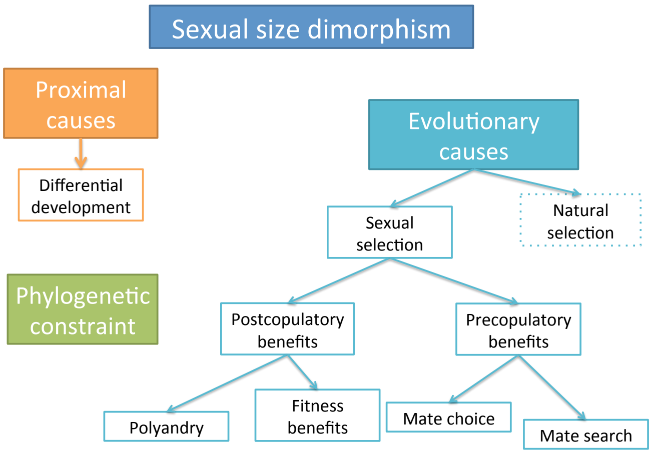

Fig. 1: Integrative approach to the understanding of sexual size dimorphism evolution.

In my first chapter, published in *Ethology*, I focus on mate choice and postcopulatory benefits of SSD, testing for fitness benefits associated to larger female size and smaller male size. My results show that mate choice and postcopulatory fitness benefits are not related to the evolution of SSD in *Mecaphesa celer.* All virgin females mated readily with the first male presented to them, showing no evidence of precopulatory mate choice. Once mated these same females became extremely aggressive towards males, but some of them accepted copulating with a second male (polyandry).

Females mating with multiple males (i.e. polyandry), even if infrequent, challenges the benefits of early access to virgin females (Wiklund & Forsberg 1991). To gain a better understanding of the role of polyandry in *M. celer*'s mating system, in my second chapter I focused on the relation between SSD and a female's decision to mate with multiple males. I found no evidence that female or male size influence the female's decision to mate with multiple males. Moreover, I found no difference in fitness between monandric and polyandric females. Interestingly, however, I found that paternity in polyandric females follows a pattern of sperm mixing, with a potential slight advantage for second males. These results, now accepted for publication at *Animal Behaviour*, confirm that mate choice and postcopulatory fitness benefits are not related to the evolution of SSD (Chelini & Hebets, *in press*).

Results from my first and second chapter provide mixed support for the hypothesis that SSD in *M. celer* is a consequence of selection for protandry (i.e. early male maturation). Although the lack of mate choice in virgin females and the short window of time during which females are receptive to second males support a scenario of scramble competition for virgin females, the fact that some females do mate with multiple males, and that this species show a pattern of sperm mixing, with a slight advantage for last males, contradict this hypothesis (Elgar 1998). In my third chapter I focus on understanding the developmental trajectories of females and males in order to test whether and how males mature earlier than females. By tracking the growth and standard metabolic rate of laboratory-born spiderlings, I found that growth rate, and not simply number of instars, underlies SSD in *M. celer*. Females show longer and faster growth, but this is not fueled by a higher metabolic rate. On the contrary, females' and males' mass-specific metabolic rates at rest fall within the same range. I also tested the influence of environmental factors, more specifically diet and temperature, on the degree of SSD of this species and found that the interaction of diet and temperature affected only female size, and not male size. I suggest that females achieve a higher growth rate through a combination of high food ingestion and low activity levels.

Finally, in in my fourth chapter, I compared the results obtained in the laboratory in my first three chapters with the population dynamics of *M. celer* in the wild. Selection for protandry may indirectly lead to SSD due to a reduction in the development time of males, but selection may also act directly on SSD, with protandry as an indirect consequence (Morbey & Ydenberg 2001). I followed a population of *M. celer* in the field through an entire reproductive season, to test if female biased SSD evolved through selection for protandry due to increased mating opportunities. *M. celer* is indeed protandric in the field, but the difference between female and male maturation time is much shorter in the field than in the laboratory, indicating a high degree of plasticity in *M. celer*'s growth rates. Contrary to what I expected, the proportion of virgin females was higher at the end of the reproductive season than at the beginning. The absolute number of virgin females, however, is likely to be higher early in the season, as all females are virgin upon maturation and *M. celer* lives for only one year. Also surprisingly, the degree of protandry isn't proportional to the degree of SSD, as late-matured females and males are smaller than early-matured ones. As such, my results provide partial support for the mating opportunity hypothesis, as early-matured males benefit from access to a higher number of virgin females.

SSD seems to be a consequence of protandry in this species, but the high plasticity in female and male growth rates challenges the assumption that SSD in spiders is simply a consequence of selection for early male maturation. My results highlight the need for integrative studies analyzing the role of multiple sources of selection leading to differences in female and male size, as well as the proximate mechanisms underlying SSD, for an in depth understanding of the evolution of sexual size dimorphism.

CHAPTER 1

Absence of mate choice and postcopulatory benefits in a species with extreme sexual size dimorphism

Abstract

Most hypotheses related to the evolution of female-biased extreme sexual size dimorphism (SSD) attribute the differences in the size of each sex to selection for reproduction, either through selection for increased female fecundity or selection for male increased mobility and faster development. Very few studies, however, have tested for direct fitness benefits associated with the latter - small male size. *Mecaphesa cele*r is a crab spider with extreme SSD, whose males are less than half the size of females and often weigh 10 times less. Here, we test the hypothesis that larger size in females and smaller size in males are sexually selected through differential pre- and postcopulatory reproductive benefits. To do so, we tested the following predictions: matings between small males and large females are more likely to occur due to mate choice; females mated to small males are less likely to accept second copulation attempts; and matings between small males and large females will result in larger clutches of longer-lived offspring. Following staged mating trials in the laboratory, we found no support for any of our predictions, suggesting that SSD in *M. celer* may not be driven by pre or post-reproductive fitness benefits to small males.

Introduction

The average body size of most animals at sexual maturation results from the delicate balance between selection for survival and selection for reproduction (Stillwell *et al.* 2010). In species with sexual size dimorphism (SSD), the size difference between the sexes can be attributed to these two sources of selection acting differently upon each sex, and reaching equilibrium at different points (SSD equilibrium model: Blanckenhorn 2005). While sexually dimorphic traits, such as male ornaments and weapons, are a classic example of strong selection for reproduction acting upon males only (Andersson & Simmons 2006), physiological constraints and genetic correlations are expected to maintain the size of both sexes within similar ranges (the null hypothesis of size equality - Hedrick & Temeles 1989; Andersson 1996). Differences in female and male sizes are, however, not only very common, but sometimes quite extreme, such as in many examples of female biased SSD (Stillwell *et al.* 2010). The evolution of such female biased SSD must reflect selection acting in opposing directions, or at least with different intensities, in males and females (Blanckenhorn 2000, 2005; Kuntner & Elgar 2014).

Selection for reproduction in females is often associated to increased egg production (Clutton-Brock & Vincent 1991; Stillwell & Davidowitz 2010a). Fecundity benefits related to a larger female body size are well documented across various animal groups (e.g. mammal: Fokidis et al 2007; birds: Sedinger et al 1995; reptiles: Hendry et al 2014; insects: Honěk, 1993 and spiders: Beck & Connor 1992, Prenter, Elwood, & Montgomery 1999). As such, selection for increased fecundity is often assumed to be the main mechanism leading to maintenance and/or exaggeration of female size in species with female biased SSD (see Blanckenhorn 2005 and references therein, Stillwell *et al*. 2010).

In contrast to our understanding of selection for increased female size, in most taxa, little is understood about how selection might act to reduce or maintain small male size (Blanckenhorn 2005). The role of selection in the reduction or maintenance of small male size is typically attributed to an increase in the chances of a male's encounter with suitable females, or a reduction in the risks associated with such encounters (Ghiselin 1974; Vollrath 1998). It is hypothesized that small males may benefit by being more agile (e.g. midges – Crompton *et al*. 2003), or by reaching sexual maturation faster than females (e.g. paedomorphic bone-worms – Rouse, Goffredi & Vrijenhoek 2004), or faster than larger male competitors (Kasumovic & Andrade 2009). Once a suitable female is encountered, small males may also benefit through opportunistic cohabitation and kleptoparasitism (e.g. golden orb-web spiders – Kasumovic *et al.* 2006).

Spiders are the only taxon where males are often less than half the size of females, with such extreme SSD having evolved independently in at least seven families (Scharff & Coddington 1997; Hormiga *et al.* 2000). Three not mutually exclusive hypotheses are commonly associated to the evolution of small male size in spiders. The first is adaptive protandry, where males mature earlier than females and benefit from minimum competition for access to sexually receptive females, being particularly adaptive in species where females mate only once and/or where there is first male sperm priority (Maklakov, Bilde & Lubin 2004; Kasumovic & Andrade 2009). The second is the differential male mortality hypothesis, which predicts that in species whose adults have distinct lifestyles (such as sedentary orb-weaving females and wandering males), small size would allow males to suffer smaller mortality risks

(Vollrath & Parker 1992; De Mas, Ribera & Moya-Laraño 2009). Finally, the adaptive sexual cannibalism hypothesis predicts that small male size increases the probability of a male being eaten by a female post-copulation, which is adaptive in species where the consumption of the male by a female immediately after copulation can increase the reproductive success of both sexes (Andrade 1996; Welke & Schneider 2010, 2012; but see Fromhage, Uhl & Schneider 2003; Foellmer & Fairbairn 2004). While many sexually cannibalistic species also exhibit extreme SSD, however, not all spider species exhibiting SSD perform sexual cannibalism (Wilder & Rypstra 2008). Interestingly, despite the abundance of work done on the evolution of SSD in spiders, very few studies have examined the hypothesis that there may be pre and/or postcopulatory fitness benefits associated with female and male size (but see (Schneider *et al.* 2000).

Crab spiders (family Thomisidae), exhibit some of the most extreme examples of SSD among animals (Legrand & Morse 2000). Females seldom cannibalize their mates, making adaptive sexual cannibalism unlikely to account for the extreme female-biased SSD observed in crab spiders. Nothing is known about size-dependent differential survival and/or mortality in this group, leaving the potential for differential male mortality wide-open. Males of several Thomisidae species are known to be protandric, maturing earlier than females (Muniappan & Chada 1970; Dodson & Beck 1993; Morse, 2013a). In some species this earlier maturation seems to favor small males given that females mate only once (e.g. Holdsworth & Morse 2000). In other species, however, females mate multiply, which could hinder the benefits associated to an early male maturation (Dodson & Beck 1993, Chelini *Chapter 2*).

In this study, we expand upon the wealth of prior work assessing SSD to test the relatively unexplored hypotheses that pre and/or postcopulatory fitness benefits select for the combination of small male size and large female size in the crab spider *Mecaphesa celer*. *Mecaphesa cele*r is a flower-dwelling crab spider specialized in preying upon pollinators. Males of this species are approximately one half the total body size of females, and often weigh less than 1/10th of the average female mass. Nothing is known about the evolutionary drivers of SSD in this species.

In this study, we aim to answer two specific questions. (1) Does female and/or male size influence the probability of mating and/or remating? (2) Does female and/or male size affect clutch size and offspring survival? If body size influences the probability of mating and/or remating and if this has influenced SSD in this species, then we predict that: (1a) small males will be more likely to obtain copulations with large females than large males and (1b) females mated to large males will be more likely to accept subsequent copulation attempts than females mated to small males. If body size influences clutch size and offspring survival and this has influenced SSD in this species, then we predict that dissortative matings according to body size– specifically between large females and small males - will result in: (2a) larger clutches and (2b) offspring that will be longer-lived than the offspring of other size combinations.

Methods

Animal collection and maintenance

We collected spiders as juveniles (3rd – 5th instar) in Oxford, MS, USA in March 2013 and Lincoln, NE, USA in May 2013. In the laboratory, we housed spiders in individual 4 x 4 x 6 cm acrylic cages, with *ad libitum* water, and small artificial plants for perching. All plants were composed of a ramified plastic stem with two flowers set 3 cm apart. All spiders were in a room at 26° C and 60% relative humidity, under a 14:10 light:dark cycle. We fed all individuals twice a week with juvenile crickets (*Acheta domesticus*, 1 mm, Ghann's Cricket Farms – GA, USA) and recorded the date of all molts.

Mating trials

We conducted all mating trials between June 3rd and July 21th 2013, from 10:00 to 17:00. Trial arenas were cylindrical 12 x 7 cm (diameter x height) acrylic tubes. We placed the artificial plant from each female's cage in the center of the arenas prior to a trial, fixed in a square 1 x 1 cm piece of plasticine. We placed each arena on top of a small square mirror on a 20 cm of diameter rotating platform, in order to be able to observe any behavior happening on the underside of the flowers without disturbing the focal individuals. We transferred females into the arenas along with their own artificial plant. Females were usually resting on top of the flower petals inside their cage, making their transfer easy. In cases where the females were not originally on top of their plants, we transferred them gently using a soft paint brush, allowing them to climb back onto the top flower once the plant was inside the experimental arena. We then allowed females to acclimate for 5 min before introducing males. We transferred males into the arena using a soft paint brush, and placed them on the stem of the artificial flower, at least 3 cm away from the females. Based on preliminary observations we determined that the decision to mate or not was made within minutes of the first contact between male and female. As such, our trials ran for 30 minutes, or until copulation was over in the cases where mating did occur.

We cleaned arenas and mirrors with 70% ethanol and used new pieces of plasticine for each trial, to eliminate potential chemical cues.

Female and male size quantification

Once all trials had been run (see *Precopulatory benefits of large female size and small male size*), we sacrificed all individuals by freezing them, and later transferred them to 75 % ethanol. To quantify female and male size, we photographed all adult individuals using a Spot Flex digital camera (Model 15.2 64 MP, Diagnostic Instruments, Inc.) mounted on a Leica DM 4000 B Microscope. Using the software ImageJ (Rasband 1997-2012) with our photographs, we took two measures of size from each adult individual: cephalothorax width (cw) and first right femur length (fl). Female and male femurs and cephalothorax widths are normally distributed variables (Shapiro-Wilk test, female cw: $w = 0.98$, $p = 0.65$; male cw: $w = 0.96$, $p = 0.13$; female fl: $w = 0.98$, $p = 0.31$; male fl: $w = 0.98$, $p = 0.12$).

Precopulatory benefits of large female size and small male size

In order to test our predictions related to the precopulatory benefits of *M. celer*'s female biased SSD, we paired each of 60 females with three distinct males, with a two-day interval between each trial. We opted for presenting males to females in a sequential order rather than simultaneously based on preliminary observations that mutual avoidance by males and a heightened state of aggressiveness of the female would often hinder copulation attempts when males were presented to the females simultaneously. Our main response variable was occurrence of copulation, but in order to describe the mating behavior of this species, we also took note of copulation duration, aggressiveness of females towards males and mate guarding of females by males. Given the small size of these animals and the fact that we did not want to manipulate individuals prior to behavioral trials, we paired females and males randomly according to size and took their measurements under the microscope only after sacrificing them (see *Female and male size quantification*, above). Females and males ages varied between 2 and 15 days post sexual maturation (average = 5 days). All females were virgin at the time of their first trial, but due to a limitation in the number of adult males available we were constrained to use some males more than once (n = 13). Virgin and non-virgin males did not differ in size (average virgin male cw = 1.48 mm, average non-virgin male cw = 1.47 mm, *t* test: t = -0.57, df = 102.12, p = 0.56; average virgin male fl = 2.89 mm, average non-virgin male fl = 2.88 mm, *t* test: t = -0.34, df = 103.04, p = 0.73).

We tested the effect of male virginity on all of our response variables using a generalized linear model (GLM), with the virginity status as the predictor variable. When male virginity had an effect on the response variable, we ran the analyses using only data from matings with virgin males. When the male virginity did not have an effect on the response variable, we used the complete dataset. To test if *M. celer*'s female biased SSD is related to precopulatory benefits, we built two sets of models – one for each prediction:

1a) Mate choice: To test if small males are more likely to obtain copulations with large females than large males, we used a generalized linear model (GLM) with a Binomial distribution and a logit link function. We used female size, male size, and the interaction between these two variables as independent variables and mating success as the binomial response variable.

1b) Probability of remating: To test the prediction that females mated to large males will be more likely to remate, we used a similar statistical approach, replacing mating success by remating success as the binomial response variable and adding the size of the second male as a predictor variable. To avoid model overfitting due to the small number of successful rematings and our large number of parameters, we ran six separate simple binomial models instead of one full model, each corresponding to one predictor variable or one interaction. For the same purposes, we treated second and third males as equivalents. We ran these analyses using the software R (R Development Core Team 2009).

Postcopulatory benefits of large female size and small male size

To test our predictions related to postcopulatory benefits of *M. celer*'s female biased SSD, we focused on the females that had mated with only one male in the above-mentioned mating trials, and quantified two potential benefits: (2a) clutch size, and (2b) spiderling survival. In order to quantify clutch and survival, we kept all females alive for up to three months after the trials, feeding them once a week. We monitored these females three times a week to record if they had laid an egg sac, and if the spiderlings had hatched from their egg sac. Once the spiderlings had hatched and dispersed from the egg sac (approximately 3 days after eclosion, Muniappan & Chada 1970), we separated them from the mothers and counted them in order to estimate spiderling number. Egg sacs that had not hatched after 60 days were counted as a failed clutch. We preserved the remaining non-developed eggs and the egg sac in ethanol 70%, and later counted these eggs using a stereomicroscope (see *Female and male size quantification*).

After counting all live spiderlings, we housed them individually under the same conditions as the adults, but in 1 x 3 cm glass vials with a 0.5 x 2 cm strip of plastic netting for perching, and checked them every two days to estimate survival.

We chose to keep the spiderlings with no food in order to assess their resistance to starvation. Resistance to starvation is often correlated with the amount of yolk contained in each egg, and therefore with female investment in a clutch (Fox & Czesak 2000). This proxy allowed us to test the hypothesis that females invest differently in their clutches according to their own size and their mate's size. We could not assess maternal investment through the mass of recently hatched young given that *M. celer* spiderlings weigh less than 0.1 milligram, making it extremely challenging to obtain a reliable measure of mass, even when averaging the mass of an entire clutch. To test if *M. celer*'s female biased SSD is related to postcopulatory benefits, we ran four analyses:

2a) Clutch size: We tested the prediction that dissortative matings between small males and large females will produce larger clutches in three steps. First, to test if large females produced more eggs than small females, particularly when mated to small males, we ran a linear model (LM), using female size, male size, and their interaction as independent variables and total number of eggs as the response variable. Then, to test the effect of female and male size on the probability of a female having a successful clutch, we ran a GLM with a Binomial distribution and a logit link function, using female size, male size, and their interaction as independent variables and clutch success as the binomial response variable. In order to avoid having overdispersion issues, and given that we had only one spiderling number outlier (a clutch of only four spiderlings), we treated this clutch as failed and included it in this analysis. Finally, to assess if female and male size affect the number of spiderlings hatching from a successful clutch, we focused on matings that had produced a successful clutch, and ran a linear model (LM), using female size, male size, and their interaction as independent variables and number of spiderlings as the response variable. We ran these analyses using the software R (R Development Core Team 2009).

2c) Spiderling survival: To test the prediction that large females and small males will produce longer-lived offspring, we ran a Mixed Effects Cox model with the functions *Surv*, *survfit* and *coxme*, of the R software library package *survival* (Therneau 2015). We used each clutch as a random variable, and female femur, male femur, their addition and their interaction as predictor variables.

Results

Precopulatory benefits of large female size and small male size

1a) Mate choice and mating behavior: All tested females (n = 60) mated with the first male introduced to them. Only one female tried to cannibalize the male, biting his leg during copulation and forcing the male down from the typical mating position (i.e. the male on the back of the female's abdomen). That particular male autotomized his leg and ran away. All females seemingly passively allowed their paired male to mount and mate them, showing no evidence of precopulatory mate choice.

Males mounted the females shortly after first touching them, with no observable stereotyped courtship behavior, and often no more than a single contact. If the female turned aggressively towards the male prior to or upon contact, the male would retreat rapidly, then slowly re-approach the female and tap on her abdomen with his first pair of legs fully extended in front of his body. Once the male touched the female's abdomen, females would accept the males' approach. Copulation started with the male climbing on the female's abdomen, then lowering his opisthosoma and front legs along the side of her body in such a manner that one of his pedipalps could be in contact with one of her genital openings, on the ventral surface of her abdomen. After the insertion of one pedipalp was complete, the male would back up onto the dorsal side of the female's abdomen, and lower himself again against her other side. Copulation lasted 22.74 minutes on average (min = 5, max = 82, sd = 14.89), and ended with the male descending from the female's abdomen and distancing himself rapidly. Four males remained on the female's back for periods between 45 minutes to 3 hours after finishing their insertions. Given that no female rejected her first mate, we did not perform any statistical analysis regarding the influence of female and male size on the probability of copulation.

1b) Probability of remating: Only nine (15%) females accepted a second copulation, four with the second male only, four with the third male only, and one with both males. Females that did not remate often reacted aggressively to the approach of the male, attacking them upon contact and sometimes biting them, either forcing males to drop legs or killing them (n = 17 attacks, 10 of which resulted in male death or leg loss). Females that did not attack males usually just moved away from them, or raised their front legs in a predatory posture without striking, and males did not pursue them.

Male virginity status did not influence the probability of remating (GLM: p = 0.89, d.f. = 55, deviance = 0.23, 1st male virginity status: p = 0.87; 2nd male virginity status: p = 0.63). Female size, male size and their interactions did not influence the probability of remating (GLMs cephalothorax width (cw): female cw: p = 0.93, d.f. = 56, 1st male cw: p = 0.52, d.f. = 56, 2nd male cw: p = 0.19, d.f = 56, female cw * 1st male cw: p = 0.24, d.f. = 54, female cw * 2nd male cw: p = 0.19, d.f = 54, 1st male cw * 2nd male cw: p = 0.59, d.f = 54; GLMs femur length (fl): female fl: p = 0.56, d.f. = 56, 1st male fl: p = 0.71, d.f. = 56, 2nd male fl: p = 0.66, d.f = 56, female fl * 1st male fl: p = 0.932, d.f. = 54, female fl * 2^{nd} male fl: p = 0.31, d.f = 54, 1^{st} male fl * 2^{nd} male fl: p = 0.60, d.f = 54).

Postcopulatory Fitness Benefits

2a) Clutch size: The total number of eggs laid by females varied from 17 to 100 (mean±s.e = 56.61±18.24). One single-mated female never laid eggs. Male virginity status significantly influenced the total of number of eggs laid (LM: F = 7.14, d.f. = 45, p = 0.01), with non-virgin males siring more spiderlings than virgin males.

Considering only the subset of virgin males (n = 34), female and male size did not affect the number of eggs laid after each mating (LM cephalothorax width: F = 0.61, d.f. = 29, p = 0.61, female cw: p = 0.22, male cw: p = 0.24, female cw * male cw = 0.22; LM femur length: F = 0.43, d.f. = 29, p = 0.74, female fl: p = 0.49, male fl: p = 0.53, female fl * male fl = 0.48).

Out of the 49 single-mated females, only four failed to lay a successful clutch: one female never laid eggs, two females laid egg sacs that never hatched, and one female laid an egg sac containing only four live spiderlings. All of these females were smaller than average, and were mated to smaller than average males. Male virginity status had no effect on the probability of laying a successful clutch (GLM: p = 0.57, d.f. = 48, deviance = 0.31). Female cephalothorax width, male cephalothorax width and their interaction affected positively the probability of having a successful clutch (GLM: d.f. = 48, residual deviance = 25.07, female cw: estimate = 112.19, p = 0.02, male cw: estimate = 164.70, p = 0.02, female cw * male cw: estimate: -73.17, p = 0.02). Female femur length, male femur length and their interaction did not affect the probability of laying a successful clutch (GLM: d.f. = 48, residual deviance = 27.48, female fl: p = 0.11, male fl: p = 0.097, female fl * male fl = 0.12).

Spiderling number ranged from 25 to 99 (median±s.e. = 58.73±2.62). One female laid two egg sacs within a month (in this case spiderling number was counted as the sum of both egg sacs). Male virginity status significantly influenced the number of spiderlings (LM: F = 11.954, d.f. = 43, p = 0.001), with non-virgin males siring more spiderlings than virgin males (Fig. 1). Considering only the subset of virgin males (n = 34), female and male size did not affect the number of spiderlings resulting from each mating (Fig. 2; LM cephalothorax width: F = 1.336, d.f. = 30, p = 0.2813, female cw: p = 0.0925, male cw: p = 0.0920, female cw * male cw = 0.1012; LM femur length: F = 0.9562, d.f. = 30, p = 0.4261, female fl: p = 0.128, male fl: p = 0.131, female fl * male fl = 0.138).

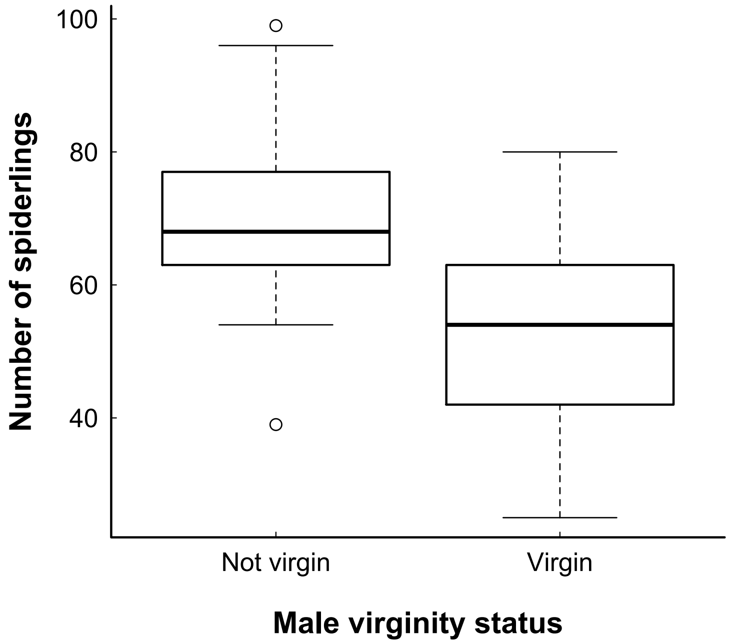

Figure 1: The relation between *Mecaphesa celer* males' virginity status and the number of spiderlings sired.

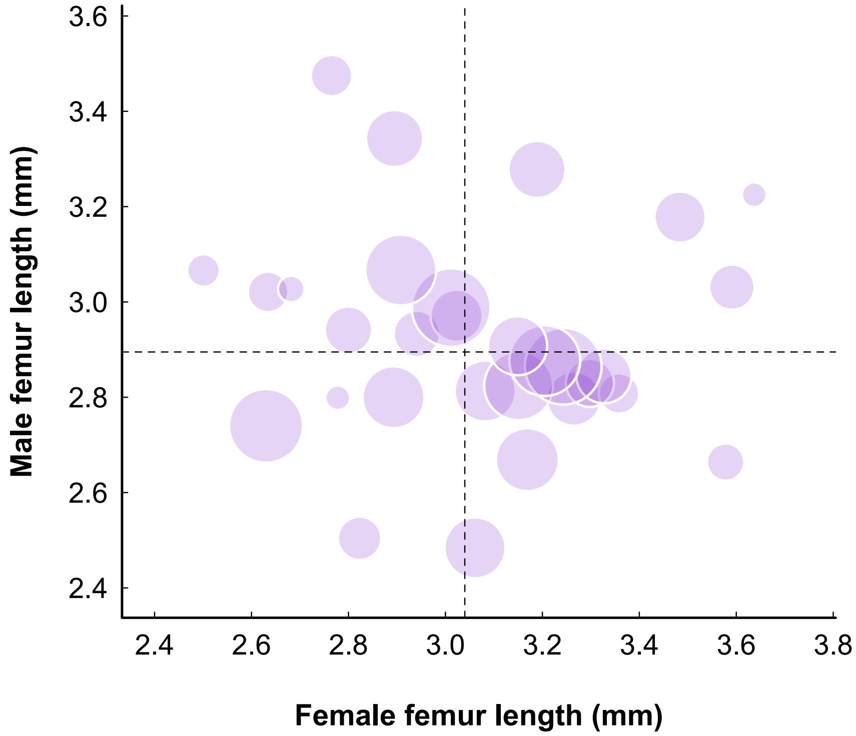

Figure 2: Number of spiderlings (represented by the diameter of the circles) in relation to female and male size. Dashed lines indicate the average size of females (vertical line) and males (horizontal lines). The bottom left and top right quadrant therefore indicate both types of assortive matings, and the bottom right and top left indicate dissortive matings.

2c) Spiderlings survival: All spiderlings died before reaching the 25th day of observation, with most losses occurring after the 9th day (average clutch survival = 15 days, min = 7, max = 23). Most clutches lost more than 50% of their spiderlings between days 9 and 13 (Fig. 3). Male virginity did not affect spiderling survival (Mixed Effects Cox Model: p = 0.5858, d.f. = 4, male virginity: p = 0.81). Male and female size did not affect spiderling survival either (Mixed Effects Cox Model cephalothorax width: p = 0.43, d.f = 3, female cephalothorax width: p = 0.79, male cephalothorax width: p = 0.88, female cephalothorax width * male cephalothorax width: p = 0.84; Mixed Effects Cox Model femur length: p = 0.09, d.f = 4, female femur: p = 0.36, male femur: p = 0.06, female femur * male femur = 0.06).

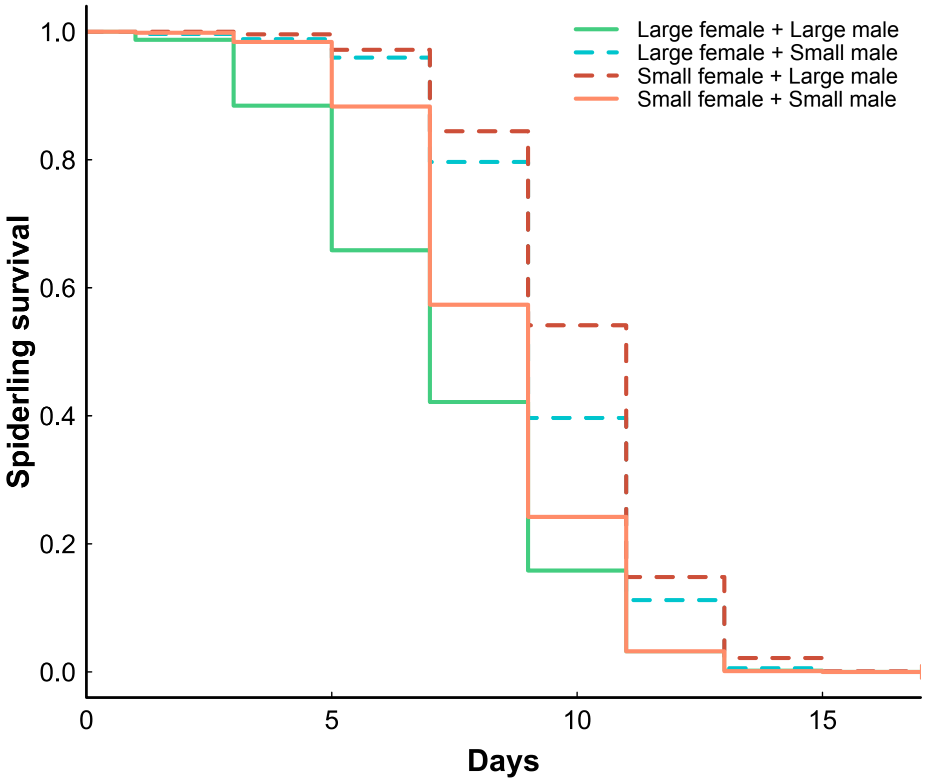

Figure 3: Survival analysis of *M. celer* spiderlings for two categories of female and male size. Solid lines represent survival of offspring from assortative matings by size; dashed lines represent survival of offspring from dissortative matings by size.

Discussion

Sexually dimorphic traits are often expected to be associated with pre- or postcopulatory fitness benefits. By testing the effect of *M. celer*'s female and male size on initial mate choice, remating behavior, clutch size and offspring survival, we found that the SSD of this species does not appear to be driven by any of our putative pre-or postcopulatory benefits. Indeed, female size, male size and their interaction had no effect on the probability of mating, the probability of remating, the number of spiderlings resulting from a copulation or on the survivorship of such spiderlings.

All of our initial mating trials resulted in a copulation. Not only did we not find any evidence that size is relevant for mate choice, we found that virgin female *M. celer* are apparently receptive to any male, suggesting an absence of virgin female mate choice in this species. Other flower dwelling crab spiders similarly seem to lack any type of mate choice (Dodson & Beck 1993, Morse 2007), but in at least one of these species (*Misumenoides formosipes* - Dodson & Schwaab 2001), males are known to engage in combat over the guard of sub-adult females. We never observed any agonistic encounter between *M. celer* males, in the laboratory or in the field, and therefore do not have any evidence that male-male competition could be the selecting mechanism leading to female-biased sexual size dimorphism in this species.

The seeming absence of virgin female mate choice in *M. celer* might be explained by their natural distribution and remating behavior. Mate choice is known to be influenced by the probability of encountering a suitable mate (Willis, Ryan & Rosenthal 2011). In populations with a low female-male encounter rate, females may benefit from securing fertilization success regardless of the quality of the first male encountered (e.g. Schafer & Uhl 2004). These females may then perform sequential mate choice, becoming more selective in future encounters, mating only with males bearing preferred traits, and cryptically selecting their sperm to fertilize their eggs, or at least benefiting from the increased genetic variability of their offspring (Schneider & Elgar 1998, Jennions & Petrie 2000, Fox & Rauter 2003). The chances of encounter for male and female *M. celer* are likely to be extremely variable in the wild, given that population density can vary from less than 1 spider/20 m^2 to more than 5 spiders/ 1 m^2 (MCChelini, *unpublished data*).

Only 15% of our females accepted a second mate, with 30% of the remaining mated females reacting aggressively to the males approach. Our data suggest that *M. celer* females become more aggressive and perhaps choosier after an initial mating. Our observed pattern of no choice for virgin females followed by relatively few second matings might suggest, as proposed earlier, that these females are securing a first copulation and then performing cryptic female choice if a better second male is encountered. The very low percentage of female that did accept a 2nd copulation, however, suggests that this is not a widespread strategy in *M. celer*. Moreover, we found no evidence that absolute or relative female and male size are related to remating probability, reinforcing the idea that mate choice, even in second matings, is not the selecting mechanisms leading to *M. celer*'s female biased SSD.

Mate-choice may be a costly mechanism, evolving only in systems where the benefits associated with selective mating exceed its costs (Kokko *et al.* 2003; Kotiaho & Puurtinen 2007). In species with relatively low encounter rates, such as *M. celer*, the simple risk of not finding a mate represents a high cost for mate choice. In the absence of pre- or postcopulatory benefits, and with mate-choice being costly, we might predict an absence of mate-choice. The lack of mate choice of *M. celer* is therefore quite parsimonious, given that we found no evidence that large female size and small male size are associated with higher postcopulatory benefits.

Female and male sizes were not related to the number of eggs laid, to the number of spiderlings, or to the survival of spiderlings. The probability of success of a clutch, however, was affected to the body size of males and females, as well as by their interaction, but not by leg length. Morse (2013) found that small *Misumena vatia* (Thomisidae) females often failed to lay clutches in the field even when mated, probably because their poor condition hindered their fecundity. When supplied with food, these females readily laid fertilized clutches. It is possible that very small *M.celer* are unable to produce clutches, however, size had no effect on the total number of eggs laid, indicating that failing to lay a successful clutch isn't related to small females being unable to produce, or lay eggs, but on the interaction between female egg production and male fertilization success. Small males have been shown to fertilize more eggs in other extreme sexually dimorphic spider species (Schneider *et al.* 2000; Elgar, Schneider & Herberstein 2000), but our results suggest that any relationship between male size and fertilization success is likely species-specific. Although this result points to an interesting interaction between female and male effects, we would like to acknowledge that our very low number of failed clutches (n = 4) and the relatively high number of parameters in our model (k = 3) may be resulting in model overfitting and Type II error. Nonetheless, these are interesting results that should be further explored in future studies with larger sample sizes.

Surprisingly, however, we found a positive relationship between male mating status and clutch size, with non-virgin males having larger clutches than virgin males. Assuming that this is a male-driven pattern, we can only speculate that given the potentially high costs of mate search, and the spatially uneven distribution of females in the wild, virgin males could be retaining sperm in their first copulation in order to be able to remate if a second female is found in a short period of time. Indeed, male crab spiders typically require a period of a few hours to a day to recharge their pedipalps with sperm before being able to remate (Morse 2007b). Interestingly, the total number of eggs laid was also affected by male virginity status, suggesting that this pattern may be at least partially female-driven. It is possible that females lay more eggs when mated to a non-virgin, more successful male. Future work is required to test these hypotheses.

The lack of relationship between female size and number of spiderlings is our most surprising result. The fecundity hypothesis for large female size (Head 1995) posits that large females should always have more offspring than small females (e.g. Beck & Connor 1992; Skow & Jakob 2003), a pattern so common that is has been considered the main driver of female biased SSD in spiders (Prenter, Elwood, & Montgomery 1999). In his classic paper challenging the fecundity advantage model, Shine (1988) proposed that the fecundity advantage model may hold only in scenarios where females are not limited energetically, and points out that a larger number of eggs in one clutch is not the same as a higher lifetime reproductive success. Although *M. celer* has only one reproductive season, females may lay up to four egg sacs following a single copulation (MCC *unpublished data)*. The fact that only one female laid a second egg sac in our experiment could suggest that our feeding regimen was too strict for these females to achieve their maximum reproductive potential; a possibility that should be followed up on in future studies.

Other exceptions to the fecundity advantage model occur when females trade quantity for quality in their clutches, investing more in each egg instead of increasing egg numbers. If large *M. celer* females were trading egg quantity with egg quality, we would expect to find an effect of female size on spiderling survival. Our results, however, show that female size, male size, and their interaction have no effect on spiderling survival, contradicting the last of our predictions. One possibility is that spiderling survival to starvation is not a good proxy of postcopulatory fitness benefits in *M. celer*. Alternatively, female and male size may indeed not be the determinants of spiderling survival. Offspring survival has been related to factors as disparate as female age (e.g. fishes: Berkeley *et al.* 2004, lizards: Olsson & Madsen 2001), past maternal condition (butterflies: Bonduriansky & Head 2007) and polyandry (crickets:

Ivy & Sakaluk 2005). As such, female and male *M. celer* may still have an effect on offspring survival, even if their influence is not relative to their size.

Altogether, our results suggest that *M. celer*'s large female size and small male size must have been selected by factors other than mate choice and postcopulatory benefits. In a species where virgin females mate indiscriminately and polyandry is relatively rare, a shortened development time is likely to benefit small males (Blanckenhorn 2000). As such, adaptive protandry is likely to be the mechanism selecting for *M. celer*'s female biased sexual size dimorphism. In depth studies on the synchrony of development of this species in the wild, as well as on the potential for sperm competition in polyandrous females are currently underway and should shed light into the evolution of this intriguing study system.

In summary, dimorphic traits are often assumed to be associated to reproductive benefits. Examples of dimorphic traits providing direct benefits are abundant, as are models associating traits that do not confer direct benefits to indirect benefits (see Jones & Ratterman 2009 and references therein). Here we present a study species that contradicts several predictions drawn from the evolutionary theory of sexual size dimorphism. In highly conserved clades, and in the absence of clear costs or benefits associated with a dimorphic trait, species may be dimorphic simply by effect of non-directional evolution (Brownian motion evolutionary model: Felsenstein 1973), as has been recently described by Cheng & Kuntner (2014) with regards to the sexual size dimorphism of the Argiopinae family of orb-weaving spiders. As such, given that SSD is common in the clade of flower dwelling crab spiders, *M. celer*'s extreme SSD may not be associated with any current pre- or postcopulatory benefit, and may simply be a consequence of relaxed selection on an ancestral character. Unfortunately, given that SSD is yet to be mapped on a family-, or even genus-wide phylogeny, we can currently only speculate on the direction and strength of selection acting upon *M. celer*'s SSD. We can also use *M. celer* as a precautionary tale against our inner bias towards studying species where the most eye-catching trait is associated to evident benefits. Our understanding of SSD evolution, and of any dimorphic trait, depends on the study and publication not only of eye-catching exceptions (see Huber 2005), but also of theory defying-cases such as this present study.

Acknowledgments

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CHAPTER 2

Polyandry in the absence of fitness benefits in a species with female biased sexual size dimorphism

Most studies exploring the evolution of female mating systems focus on species in which females are either monandric (mate with a single male) or highly polyandric (mate with multiple males), but less is understood about variation in mating decisions within a species. How and why do females of a single species decide whether or not to copulate with additional mates? In this study we attempt to answer this question in the highly dimorphic crab spider, *Mecaphesa celer*, whose females may be either monandric or polyandric. We tested three hypotheses: (1) a female's decision to remate is based on sequential mate choice; (2) a female's decision to remate has fitness consequences, with polyandry providing increased benefits; and (3) mating order predicts male paternity, following a pattern of first male sperm precedence. We conducted double-mating trials between females and males of varied sizes and age, quantified six putative fitness benefits obtained by monandric and polyandric females, and tested sperm precedence patterns using the sterile male technique. We found no evidence that female *M. celer* are performing sequential mate choice. Moreover, we found no difference in fitness between monandric and polyandric females. Finally, we found that paternity in polyandric females follows a pattern of sperm mixing. *Mecaphesa celer* females' decisions regarding mating with multiple males do not appear to be influenced by comparisons of male attributes or by future fitness benefits. We recommend future studies examining male ejaculate components that might influence female mating decisions.

Introduction

Female mating strategies select for traits that extend far beyond the conspicuous secondary sexual characteristics typically associated with sexual selection (Kvarnemo & Simmons 2013; Parker & Birkhead 2013; Pizzari & Wedell 2013; Boulton & Shuker 2015; Bocedi & Reid 2016; Yasui & Garcia-Gonzalez 2016). The number of mates a female accepts is expected to depend on the costs and benefits of each additional copulation (Emlen & Oring 1977; Hubbell & Johnson 1987; Pizzari & Wedell 2013). These costs and benefits are often mediated by the males' mating strategies (Parker & Birkhead 2013), which in turn are affected by the females' decision to mate multiply, leading to complex evolutionary feedbacks between the sexes (Wade & Arnold 1980; Kvarnemo & Simmons 2013). To understand the evolution of female mating strategies we must thus explore not only the costs and benefits females obtain from copulations, but also how their mating decisions mediate, and are mediated by, the mating strategy of the males.

For females, the costs of copulating with multiple partners (i.e. polyandry) include reduced reproductive success, infanticide (e.g. Schneider & Lubin 1997; Maklakov *et al.* 2005), physical harm (e.g. Blanckenhorn *et al.* 2002) and decreased female longevity (Arnqvist & Nilsson 2000), among others. Given that monandrous females (i.e. females mated to a single male) receive sperm from only one male, monandrous species are often characterized by high levels of precopulatory mate-choice and/or male-male competition (Emlen & Oring 1977; Kvarnemo & Simmons 2013). Benefits of polyandry are also varied (see reviews by Arnqvist & Nilsson 2000; Kvarnemo & Simmons 2013), and include increased female longevity and reproductive success (Wagner *et al.* 2001; Worthington & Kelly 2016), increased genetic variability of the brood (Zeh & Zeh 2001), larger and faster growing offspring (Watson 1998), and/or sequential mate-choice through acquisition of sperm from more attractive and/or genetically more compatible males (trading-up strategies: Watson 1991; Schneider & Elgar 1998; Jennions & Petrie 2000). Benefits of polyandry are likely to be influenced by sperm precedence patterns (Elgar 1998; Simmons 2005), and may lead to cryptic female choice, or strategic mating decisions in terms of order of mating partners (Simmons & Beveridge 2010; Kvarnemo & Simmons 2013).

Male mating strategies may evolve in response to the intensity of postcopulatory competition and to the species' sperm-precedence pattern (Wade & Arnold 1980; Simmons & Beveridge 2010 and references therein). Males from species with first male sperm precedence, for instance, may develop faster and compete over the access for virgin females (Singer 1982; Dodson & Beck 1993; Zonneveld 1996; Huber 2005; Kasumovic & Andrade 2009). This earlier male development (protandry) often results in a male-biased operational sex-ratio and female-biased sexual size dimorphism (Vollrath & Parker 1992; Vollrath 1998; Danielson-François *et al.* 2012, but see Wiklund, Nylin & Forsberg 1991; Legrand & Morse 2000). In species with some degree of sperm mixing, however, polyandry may hinder the benefits obtained by early-matured males (Birkhead & Møller 1998).

Much theoretical and empirical research has been conducted on species where females are predominantly monandric or polyandric, but less attention has been paid to variation in mating decisions within a species (but see Boulton & Shuker 2015). How do females of a single species decide whether or not to copulate with additional mates? Why do some females in a population exhibit monandry while others exhibit polyandry?

Females of the flower-dwelling crab spider *Mecaphesa celer* (family Thomisidae) appear to be both monandrous and polyandrous: some females accept only one partner over their life, but others remate with a second male (Chelini & Hebets 2016). The temporal window of receptivity to additional copulations is small in this species, and females become increasingly aggressive towards males following their initial copulation. While approximately 85% of females are willing to remate immediately after their first copulation (Chelini, *pers. obs* – N = 40.), only 15% of the females are willing to remate after two days, and approximately 5% of the females still remate after four days (Chelini & Hebets 2016). In contrast to what we might expect in a monandrous mating system, *M. celer* females do not exhibit any form of mate choice while virgin (Chelini & Hebets 2016, see also Morse 2010), and, unlike other closely related crab spiders (Dodson & Schwaab 2001), males do not appear to enter in direct contests (Chelini, *pers. obs*). Additionally, *M. celer* males are known to mate with multiple females if given the opportunity (Muniappan & Chada 1970; Chelini & Hebets 2016).

Mecaphesa celer crab spiders are also sexually dimorphic, with females approximately twice the body size of males (Dondale & Redner 1978) and often weighing more than 10 times the average male mass (Chelini, *unpublished data*) . The female-biased sexual size dimorphism observed in *M. celer* appears to be at least partly driven by early male maturation (Muniappan & Chada 1970; see also Maklakov, Bilde, & Lubin 2004; Danielson-François *et al.* 2012). It has been demonstrated that female and male body sizes do not influence the likelihood of first copulations, as all virgin females mate with the male to which they are first presented (Chelini & Hebets 2016).

In this study we ask three explicit questions: (1) What factors influence *M. celer* females' decision to copulate with a second mate?; (2) Do females receive fitness benefits from copulating with a second male?; and (3) What is the pattern of sperm precedence in this species? Given the lack of female choice in first copulations (Chelini & Hebets 2016), we hypothesize that a female's decision to remate is based on sequential mate choice (i.e. trading-up hypothesis: Schneider & Elgar 1998; Jennions & Petrie 2000), and as such depends on the comparison between the first and second male they encounter (Hypothesis 1). We also hypothesize that polyandry provides fitness benefits to females (Hypothesis 2). Finally, given the observed early male maturation (Muniappan & Chada 1970), we hypothesize that male paternity is dependent on mating order (Hypothesis 3) and predict a pattern of first male sperm precedence.

Methods

Animal collection and maintenance

We collected male and female *Mecaphesa celer* as juveniles and sub-adults (5th – 7th instar) at Holmes Lake Park, Lincoln - NE, USA in June 2014. In the laboratory, we housed spiders in individual 4 x 4 x 6 cm acrylic cages with the internal walls covered in plastic netting, allowing spiders to climb and perch. All spiders were in a room held at 26° C and 60% relative humidity, under a 14:10 light:dark cycle, with *ad libitum* water. We fed all individuals two juvenile crickets (*Acheta domesticus*, 1 mm, Ghann's Cricket Farms – GA, USA) twice a week and recorded the date of all molts including the date of maturity. No ethical approval was required for the study.

Hypothesis 1- Female decision to remate is based on sequential mate choice

Predictions

We tested three predictions of hypothesis 1:

- Prediction 1a) A *female's decision to remate is based on the first and second males' body size*. If female *M. celer* remate only if the second male is somehow superior to the first male, we predict that females mated with males of similar size would not remate, while females paired with males of very different sizes would. In the closely related *Misumena vatia*, and *Misumenoides formosipes*, larger males tend to win more male-male contests (Dodson & Schwaab 2001; Legrand & Morse 2000), and are faster and more agile than small males (Morse 2013b), an advantage likely to be important in a system with sedentary females and intense male mate search. As such, we predict that the highest remating rates will occur when the second male has a larger cephalothorax width (our chosen measure of body size) than the first male.
- Prediction 1b) A *female's decision to remate is based on the first and second males' leg length*. Despite being extremely sexually dimorphic in body size, female and male *M. celer* have similarly long legs. Prior to copulation, *M. celer* males typically touch the female's abdomen from afar with their first and second pairs of legs. Females respond to this first contact either passively, folding their legs close to their body, or with an attack (Chelini & Hebets 2016). Longer legs may therefore confer an advantage to males by allowing them to touch the female from further away, minimizing the exposure of their vital body parts to a potentially aggressive female. As such, while both cephalothorax width and femur length are measures of size, they may potentially convey different information to the female.
- Prediction 1c) A *female's decision to remate is based on the first and second males' age (in days post maturation)*. Age is known to affect female receptivity in

many species (e.g. Moore & Moore 2001; Mack, Priest & Promislow 2003; Wilgers & Hebets 2012). In addition, male age may be inversely related to the amount and quality of their sperm (Radwan 2003; Jones & Elgar 2004). In the closely related *Misumena vatia*, older males are more often rejected and cannibalized than younger males (Morse & Hu 2004). As such, we expect older *M. celer* females to be less receptive to second males than young females, and we expect females to be polyandric when the first male presented to them is older than the second one.

Mating trials and data collection

We conducted a total of 77 double mating trials between July 8th and July 29th 2014, from 10:00 to 17:00. We measured all spiders with a caliper immediately post-maturation (at least 3 days prior to a trial). We then paired our spiders in a manner such that females over the entire size range were paired with all possible combinations of first and second males' sizes. Given the small size of these animals and the difficulty of accurately measuring them while alive, we conducted our statistical analyses using measurements taken under the microscope after all spiders were sacrificed (see *Female and male size quantification*, below). There was no difference in the average size of first and second males (t test: $t = -0.61$, d.f. = 127.56, p = 0.54).

Trial arenas were cylindrical 12 x 7 cm (diameter x height) acrylic tubes. We placed an artificial plant composed of a ramified plastic stem with two flowers set 3 cm apart in the center of the arenas prior to each trial, fixing them in a 1 cm^3 piece of plasticine. To observe behaviour happening on the underside of the flowers without disturbing the focal individuals, we placed each arena on top of a small square mirror set on top of a rotating platform (20 cm dia.).

We transferred females gently from their cages to the top of the artificial flower using a soft paintbrush, and allowed them to acclimate for 20 min before introducing males. All females were virgin, and had matured in the three to 25 days prior to being tested. We transferred males in the same manner, and placed them at the base of the artificial flower, at least 5 cm away from the females. Males typically climbed on the flower stem immediately, quickly reaching the female.

As with the females, all males matured in the laboratory and were virgin. *M. celer* males mature much earlier than females (Muniappan & Chada 1970), so mating trials were run 15-50 days after male maturation. Our trials ran for 30 min, or until copulation ended for trials in which matings occurred. We cleaned arenas and mirrors with 70% ethanol and used new pieces of plasticine for each trial to eliminate potential chemical cues.

Immediately following the end of each initial trial (*i.e.* after 30 min when no copulation happened or after female and male stopped copulating and distanced themselves), we left females alone in the arenas for 2 h, and then introduced a second virgin male using a soft paintbrush. The pair was then allowed to interact for 30 min or until copulation ended. For each initial and second mating trial, we recorded the occurrence of copulation and aggressiveness of females towards males (e.g. attacking, biting, killing).

Female and male size quantification

Female mass in spiders may vary rapidly according to their feeding schedule, but measurements of structural size, such as cephalothorax width or leg length, are fixed at maturity. As such, cephalothorax width is the most commonly used proxy for size in studies with sexually size dimorphic spiders (Foellmer & Moya-Laraño 2007).

After sacrificing all adult individuals by freezing (the most ethical method of sacrificing spiders), and transferring them to 75% ethanol we photographed them using a Spot Flex digital camera (Model 15.2 64 MP, Diagnostic Instruments, Inc.) mounted on a Leica DM 4000 B Microscope. Cephalothorax width and femur length of all adults were then measured on the photographs using the software ImageJ (Rasband 1997-2012).

Statistical analyses

- Prediction 1a) A *female's decision to remate is based on the first and second males' body size*. To test this prediction we used a binomial generalized linear model (GLM) with logit-link function to account for the binomial dependent variable (remated/non-remated). We used female, first and second male cephalothorax width, and the interaction between these three variables as independent variables, and conducted a step-wise regression to select the simplest and best fit model possible. In order to minimize the number of parameters involved in the model, we repeated this analysis using multiple indexes of similarity between first and second males' sizes: $\frac{1^{st}}{2^{nd}}$; $1^{st} - 2^{nd}$, and indexes adapted from two indexes of sexual size dimorphism (Lovich & Gibbons 1992; Smith 1999). All yielded results similar to the GLM described above (data not shown), and thus we report only the GLM including female, first male and second male cephalothorax width.
- Prediction 1b) A *female's decision to remate is based on the first and second males' leg length*. We tested for the effect of male leg length with an approach identical to that of 1a), but replacing female, first male and second male cephalothorax width by femur length.

- Prediction 1c) A *female's decision to remate is based on the first and second males' age (in days post maturation)*. We tested the effect of female, first and second male age using the same approach described in 1a), replacing female, 1st male and 2nd male cephalothorax width with days post-maturation.

As our trials occurred over three weeks, we had a strong correlation between female and male age and the days elapsed along *M. celer*'s reproductive season (which in Nebraska falls between early June and early August – *pers. obs.*). To test for any effect of the timing of trials within the reproductive season, we ran another binomial GLM using trial delay (i.e. number of days elapsed between June 14th, when the first mature female was found, and the trial date) as the only independent variable.

Due to the large window of time during which females were tested, we repeated all analyses included in this manuscript using age as a covariate, but our results remained unchanged (data not shown). We ran the analyses of predictions 1a) – 1c) using the software R (R Development Core Team 2009) and the function *glm*.

Hypothesis 2) Polyandry provides fitness benefits to females

Predictions and fitness proxies

In order to test the hypothesis that females accrue reproductive benefits from being polyandric, we collected data on six different proxies of fitness benefits that females could obtain from the time period immediately following copulation until after offspring dispersal.

- Prediction 2a) *Polyandric females lay eggs faster than monandric females.* Polyandry may benefit females through the transference of nutritious seminal products and/or hormones that accelerate oviposition rate (Perry, Sirot & Wigby

2013, see review by Arnqvist & Nilsson 2000). As such, our first fitness proxy was the number of days between copulation and egg sac laying.

- Prediction 2b) *Polyandric females' eggs hatched faster than monandric females' eggs.* Following the argument presented above, double-mated females may also benefit from faster-developing embryos, minimizing the time spent caring for each egg sac and optimizing the female's chances of laying multiple egg sacs in a single season. The number of days between egg sac laying and egg sac hatching was therefore our second fitness proxy.

- Prediction 2c) *Polyandric females have higher lifelong reproductive success than monandric females.* Increased reproductive success is one of the most common benefits of polyandry (e.g. Newcomer *et al.* 1999; Arnqvist & Nilsson 2000; Wagner *et al.* 2001; Fedorka & Mousseau 2002; McNamara *et al.* 2007) . The total number of spiderlings produced by a female over her lifetime and across all of her egg sacs was our third fitness proxy.

- Prediction 2d) *Polyandric females have higher numbers of spiderlings in their first egg sac only*. Not all females lay multiple egg sacs and food availability is known to influence the number of egg sacs laid by *M. celer* (see Chelini & Hebets 2016). In the field, food availability is likely to decrease abruptly towards the end of the season (i.e. late summer/early fall) so females may invest more heavily in their first clutch than in subsequent ones. As such, the putative benefits of polyandry may be quantifiable only in *M. celer* females' first egg sac, making this our fourth fitness proxy.

- Prediction 2e) *Polyandric females will have a higher fertilization success than monandric females.* If mating with more than one male allows females to minimize risks of genetic incompatibility (e.g. Yasui & Garcia-Gonzalez 2016), we expect double-mated females to have a higher clutch success (*i.e.* the percentage of eggs fertilized in each clutch) than single-mated females.

- Prediction 2f) *Polyandric females will have spiderlings more resistant to starvation than monandric females*. Mating with multiple males may allow females to have larger and longer-lived offspring (e.g. Watson 1998). As *M. celer* spiderlings are extremely small, we were unable to obtain accurate measurements of their mass at birth. Mass at birth, is extremely correlated with spiderling survival to starvation (Walker, Rypstra & Marshall 2003), being highly dependent on the amount of yolk allocated to each egg by the female. Thus, the number of days spiderlings survived to starvation was our last fitness proxy.

Data collection

To obtain data regarding a) the time to lay egg sacs and b) the egg hatching time, we fed and monitored all females three times per week following copulations to record egg sac deposition and hatching dates. To determine c) the number of spiderlings each female had over their lifetime and d) in their first egg sac, we separated and counted the spiderlings once they had hatched and dispersed from the egg sac (approximately 3-5 days after eclosion, Chelini & Hebets 2016; Muniappan & Chada 1970). To acquire data on e) fertilization success, we preserved the remainder of each egg sac in 70% ethanol and later counted all undeveloped eggs under a Leica DM 4000 B Microscope in order to calculate the percentage of eggs fertilized in each clutch. Finally, to determine f) number of days spiderlings could survive starvation conditions, we separated out ten spiderlings from each female's first clutch and housed them individually in 3 cm tall x 1 cm diameter cages, with *ad libitum* water. We monitored these spiderlings approximately every two days, recording the date of all deaths. We sacrificed all remaining spiderlings by freezing them, and later preserved them in 70% ethanol.

Statistical analyses

- Prediction 2a) *Polyandric females lay eggs faster than monandric females.* We ran a Quasipoisson GLM with the number of successful copulations as the independent variables and the number of days between copulation and egg sac laying as the dependent variable.
- Prediction 2b) *Polyandric females' eggs hatched faster than monandric females' eggs.* We tested this prediction by repeating the analysis described in 2a), but replacing the number of days between copulation and egg sac laying by the number of days between copulation and egg sac hatching as the dependent variable.
- Prediction 2c) *Polyandric females have higher lifelong reproductive success than monandric females.* We tested this prediction with a linear model (LM), using the total number of spiderlings of each female as the dependent variable and the number of successful copulations (single-mated/remated) as the independent variable.
- Prediction 2d) *Polyandric females have higher numbers of spiderlings in their first egg sac only.* We repeated the analysis described in 2c), but focusing only on the number of spiderlings hatching from the first egg sac laid by each female.
- Prediction 2e) *Polyandric females will have a higher fertilization success than monandric females.* We ran a Quasibinomial GLM with the ratio of total number of spiderlings/total number of eggs laid by each female (from here on referred to as "clutch success") as the dependent variable and the number of successful copulations as the independent variable. We tested predictions 2a) – 2e) using the software R (R Development Core Team 2009) and the function *glm*.

- Prediction 2f) *Polyandric females will have spiderlings more resistant to starvation than monandric females.* We ran a Mixed Effects Cox model with the functions *Surv*, *survfit* and *coxme*, of the R software library package *survival*. We used each clutch (i.e. each female) as a random variable, and the female's mating status (single vs double-mated) as the independent variable.

Hypothesis 3) Male paternity is dependent on mating order

Male sterilization and mating trials

We used the sterile male technique to determine sperm priority patterns (Parker 1970; Boorman & Parker 1976; Schneider & Lesmono 2009; Magris, Wignall & Herberstein 2015). We collected penultimate males and juvenile females in late June 2015 and housed them in conditions identical to those described above (see *Animal collection and maintenance*). Once all females had matured, we sterilized 60 males through exposure to 1500 rads of X-ray irradiation, using a RADSOURCE RS2000 irradiator® (12.5 min at 120 rads/min). Two days after irradiating these males, we conducted a new round of double-mating trials following the methods described above (see *Mating trials*), but reducing the interval between males from 2 hs to 20 min, in order to maximize our chances of obtaining double-mated females. Females were sorted into four treatments: irradiated male followed by normal male (n = 25); normal male followed by irradiated males (n = 25); two irradiated males (n= 10) and two normal males (n = 10). After all mating trials had been conducted we sacrificed all males and allowed females to lay egg sacs until their natural death We estimated male paternity share through egg development and differences in spiderling numbers (Boorman & Parker 1976; Schneider & Lesmono 2009). We obtained spiderling numbers following the same methods described above (Hypothesis 2 - *Data collection*).

Statistical analyses

To test our prediction of first male sperm precedence (i.e. that first males would fertilize the majority of the eggs), we ran two analyses. First, we ran a GLM with a Quasipoisson distribution using the total number of spiderlings as the dependent variable and the male order as the independent variable, followed by a Tukey contrasts test for multiple comparisons of means. Second, to test for differences in the percentage of fertilized eggs laid in the female's first egg sac only, we repeated the same analysis using the number of spiderlings from the first egg sac only as the dependent variable.

Results

1) Female decision to remate

Males always attempted to copulate, regardless of the females' mating status (virgin versus previously mated), showing no evidence of male mate-choice. Similarly, all females mated with the first male presented to them, indicating a lack of virgin female mate choice. One female attacked the first male, subsequently accepted his approach, but killed him post-copulation. Out of the 74 trials included here, 46 females accepted a second copulation (65% - Fig. 1). Although mated females showed more aggressive behaviours than virgins, few females attempted to cannibalize their mates: 10 females attacked the second male prior to copulation, and two females remated and later killed the second male post-copulation.

The model best fit to explain the effect of cephalothorax width on the females' decision to mate multiply was the full model containing the triple interaction between female, first and second male cephalothorax width. However, even in this best fit model we found no effect of female cephalothorax width, first male cephalothorax width, second male cephalothorax width or their interaction on the females' decision to remate (Table 1). None of the indexes of similarity between males had an effect on the probability of remating (data not shown). Female and male femur length also had no effect on the probability of females mating multiply (Table 2), and neither did female and male age (Table 3). Finally, the days elapsed along the season did not influence the female's decision to mate multiply (z = 0.018, d.f. = 2, p = 0.985, deviance = 0.00032).

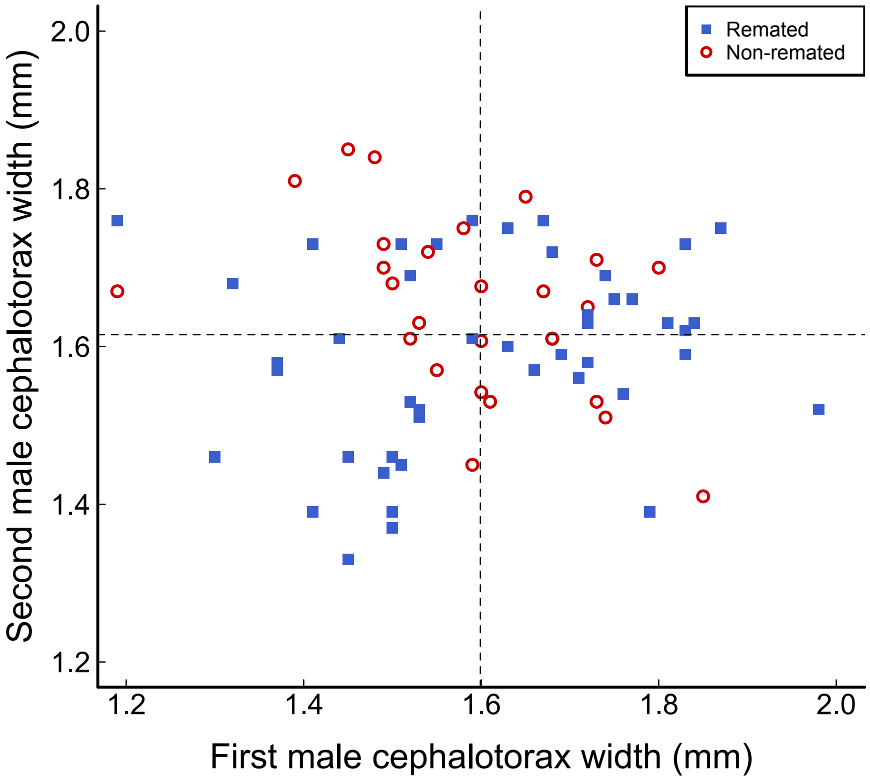

Figure 1: Female *Mecaphesa celer* that did or did not accept a second copulation, according to the size of the first and second male presented to them. All females

mated with the first male. The vertical dashed line represents the average cephalothorax width of first males and the horizontal line the average cephalothorax width of second males.

Table 1: Binomial GLM on females' decision to remate based on female, 1st male and 2nd male cephalothorax width (n = 72, Chi-square goodness of fit test: Deviance = 14.92, d.f. = 7, p = 0.04).

Parameter	Estimate	s.e.	*z* value	P value
Intercept	1046.20	685.10	1.53	0.13
Female ceph. width	-451.80	315.20	-1.43	0.15
1st Male ceph. width	-729.40	452.10	-1.61	0.11
2nd Male ceph. width	-625.40	422.10	-1.48	0.14
Female * 1st male ceph. width	320.00	208.60	1.53	0.12
Female * 2nd male ceph. width	269.60	194.00	1.39	0.16
1st male * 2nd male ceph. Width	438.20	278.20	1.57	0.11
Female * 1st male * 2nd male ceph. width	-191.90	128.20	-1.50	0.13

Table 2: Binomial GLM on females' decision to remate based on female, 1st male and 2nd male femur lengths (n = 72, Deviance = 6.69, d.f. = 7, p = 0.46).

Parameter	Estimate	s.e.	*z* value	P value
Intercept	-93.43	400.81	-0.23	0.82
Female femur length	52.22	137.70	0.38	0.70
1st Male femur length	29.27	122.44	0.24	0.81
2nd Male femur length	28.16	126.04	0.22	0.82
Female * 1st male femur length	-16.02	42.10	-0.38	0.70
Female * 2nd male femur length	-15.99	43.26	-0.37	0.71
1st male * 2nd male femur length	-8.72	38.51	-0.23	0.82
Female * 1st male * 2nd male femur length	4.88	13.23	0.37	0.71

Table 3: Binomial GLM on females' decision to remate based on female, 1st male and 2nd male ages (n = 69, Deviance = 4.85, d.f. = 7, p = 0.74).

Parameter	Estimate	s.e.	*z* value	P value
Intercept	16.26	16.44	0.99	0.32
Female age	-1.30	0.92	-1.49	0.16
1st Male age	-0.46	0.49	-0.94	0.35
2nd Male age	-0.42	0.41	-1.00	0.32
Female * 1st male ages	0.04	0.03	1.42	0.16
Female * 2nd male ages	0.03	0.02	1.43	0.15
1st Male * 2nd ale ages	0.01	0.01	1.02	0.31
Female * 1st male * 2nd male ages	-0.01	0.00	-1.45	0.15

2) Female fitness consequences of polyandry

2a) *Time to lay eggs:* Females took between 9 and 75 days to lay their first egg sac (mean = 20.32, s.d. = 10.33). Polyandry did not affect the number of days elapsed between copulation and egg sac laying (t = 1.27, d.f. = 1, p = 0.21, s.e. = 0.13, Deviance = 8.13, estimate = 0.17).

2b) *Time for egg sacs to hatch:* Once laid, egg sacs took between four and 21 days to hatch (mean = 14.34, s.d. = 2.98). Polyandry also had no influence on the number of days necessary for the first egg sac to hatch (t = -0.191, d.f. = 1, p = 0.849, s.e. = 0.054, Deviance = 0.33, estimate = -0.01).

2c) *Lifelong reproductive success:* Females laid between 25 and 154 eggs over their lifetime (mean = 80, s.d.= 33.71), spread across an average of 1.9 egg sacs (min = 1 – max = 3). Seven females never laid a successful clutch. Polyandry did not affect the life-long reproductive success of *M. celer* females (F = 1.77, d.f. = 63, p = 0.19, estimate = -11.578, Fig. 2).

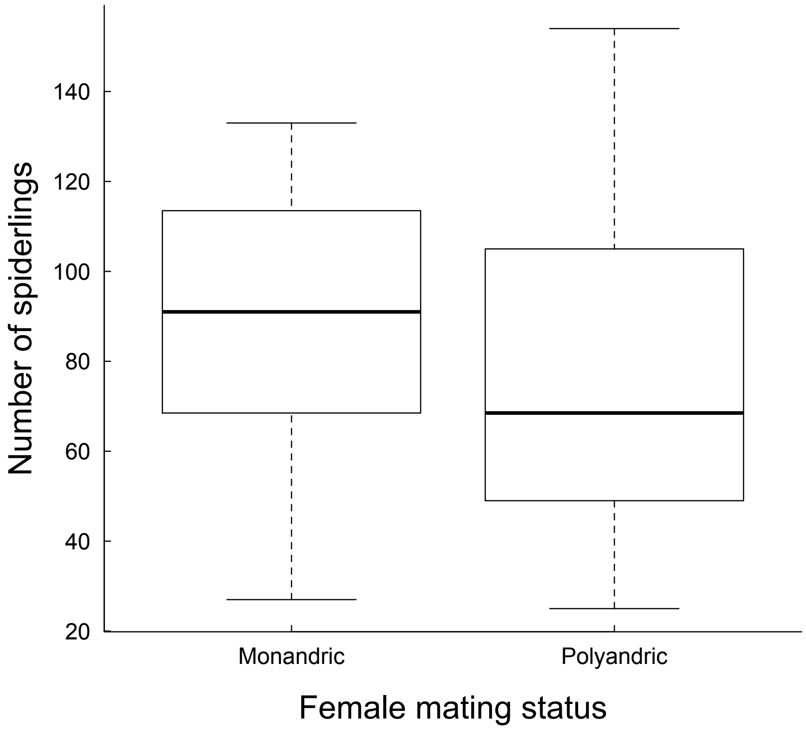

Figure 2: Life-long reproductive success of monandric and polyandric female *Mecaphesa celer*. Boxes correspond to 1st quartile, median and 3rd quartile, whiskers correspond to the range.

2d) *Spiderlings in first clutch*: An average of 51.38 spiderlings hatched from *M. celer* females' first egg sac (min = 18 – max = 95, sd = 20.07). Polyandry did not influence the number of spiderlings hatching from *M. celer* females' first egg sac (F = 1.047, d.f. = 63, p = 0.31, estimate = -5.33).

2e) *Lifelong clutch success*: The overall clutch success varied from 64% to 100% (mean = 97%, s.d. = 0.06). This proportion was not affected by the number of mates each female had ($t = 0.41$, d.f. = 63, p = 0.68, s.e. = 0.49. Deviance = -2.25, estimate = 0.20).

2f) *Spiderling survival:* Polyandry did not significantly influence the survival of *M. celer*'s spiderlings (Coxme survival model: z = 1.51, p = 0.13, coefficient = 0.43, hazard (exp(coef)) = 1.54, s.e. = 1.28, Fig. 3).

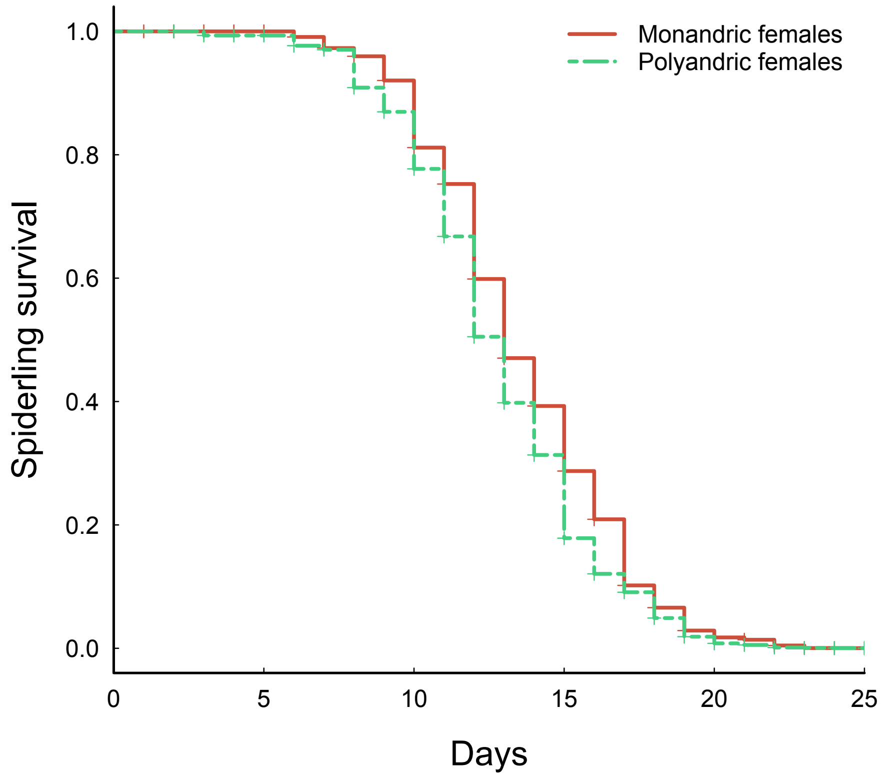

Figure 3: Average survival curves of spiderlings hatched from clutches produced by monandric and polyandric *Mecaphesa celer* females. Polyandry does not affect *M. celer*'s spiderling survival.

3) Male paternity and sperm precedence patterns

Remating rates in all treatments ranged from 75% to 85%. None of the females that mated only with irradiated males produced live spiderlings, indicating that our sterilization treatment was successful. Females in the normal-irradiated treatment produced significantly fewer spiderlings than females in the normal-normal control, but showed only a non-significant trend towards producing fewer spiderlings than those in the irradiated-normal treatment (Fig. 4, Table 4). We found no difference in the number of spiderlings produced by normal-normal control females and irradiated-normal females (Table 4). Contrary to the females' lifetime reproductive success, the number of spiderlings hatching from the first egg sac was not influenced by the males' irradiation treatment (Fig. 5 - Quasipoisson GLM: F = 1.94, Deviance = 116.1, d.f. 42, p = 0.15).

Table 4: Quasipoisson GLM followed by Tukey post-hoc comparison test on the number of spiderlings hatching from the first egg sac of females mated to males sterilized by irradiation and normal males in all possible combinations (n = 45, F = 3.42, d.f. = 2, p = 0.04, Deviance = 281.51).

Parameter	Estimate	s.e.	*z* value	P value
normal-irradiated - irradiated-normal	-0.86	0.41	- 2.11	0.08
normal-normal - irradiated-normal	0.19	0.36	0.54	0.85
normal-normal - normal-irradiated	1.05	0.46	2.28	0.05

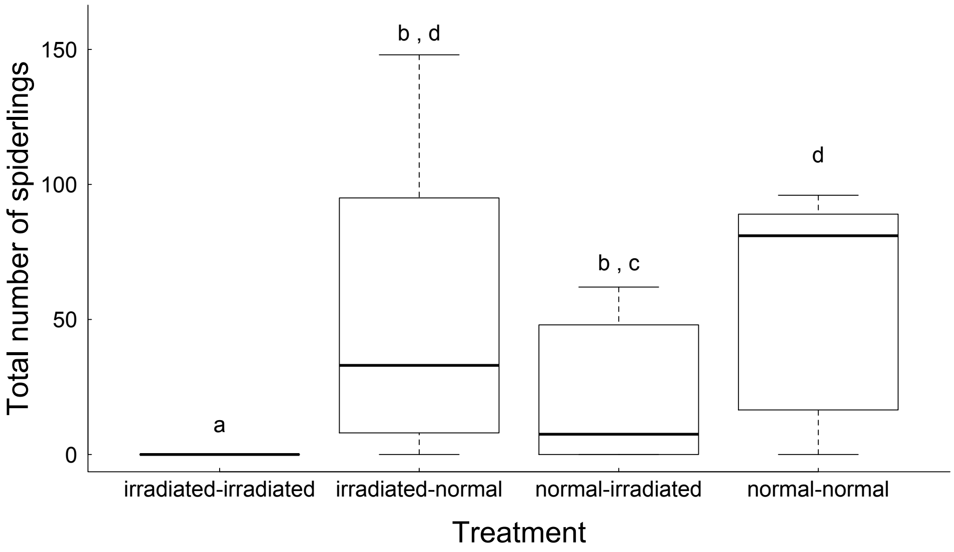

Figure 4: Total number of spiderlings resulting from matings between *Mecaphesa celer* females and two males, some sterilized though X-ray irradiation, and some normal, in all possible combinations. Different letters represent statistically significant differences. Females mated to normal males first and irradiated males second produced fewer spiderlings than females mated to two normal males (p = 0.05). Females mated to an irradiated male first and normal male second tended to produce more spiderlings than those in the normal-irradiated treatment (p = 0.08).

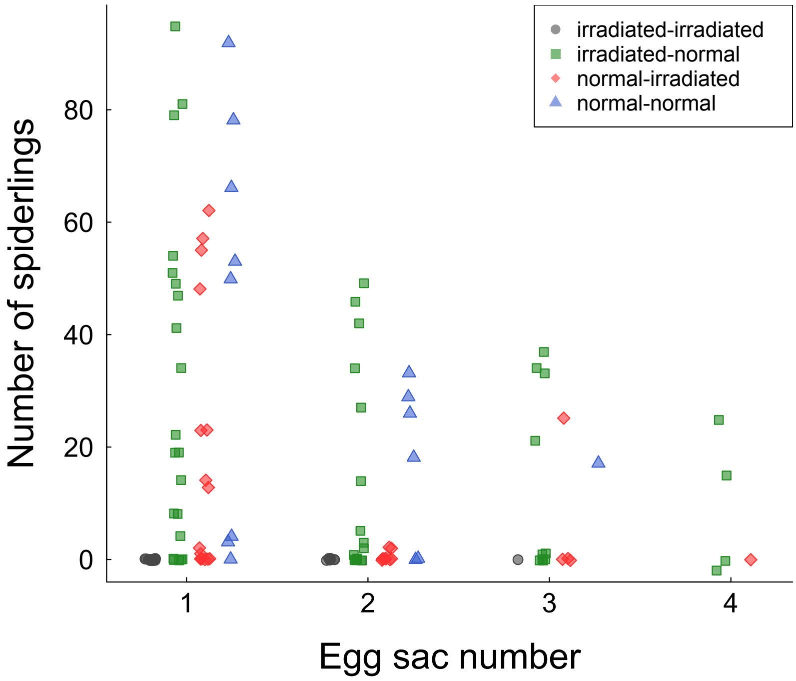

Fig. 5: Number of spiderlings hatching from each *Mecaphesa celer* egg sac, color codded by male irradiation treatment. Females mated to two irradiated males never had any spiderling hatching from their clutches. Only one female mated to a normal male followed by an irradiated male laid more than a single successful clutch, while females mated to irradiated males followed by normal males laid up to four successful clutches.

Discussion

Despite the fact that some female *M. celer* crab spiders make the decision to copulate with a second male (approximately 65% are polyandric within a 2 hour window), we did not find any evidence that these decisions were based on either male physical attributes or on female fitness outcomes. We were unable to identify any factors that influence *M. celer* females' decision to copulate with a second mate, as female remating was independent of female and/or male size, age and time along the season. Our results also show that male *M. celer* do not discriminate between virgin and previously mated females. We were also unable to identify any fitness benefits females might receive from copulating with a second male. In terms of the pattern of sperm precedence in *M. celer,* our irradiation results suggest a pattern of sperm mixing in first egg sacs, and a potential for last male sperm priority in subsequent egg sacs. This last finding reinforces the growing body of evidence indicating that the relationships between morphology, behaviour and sperm precedence patterns are complex and require direct testing (Elgar 1998; Huber 2005; Herberstein *et al.* 2011).

Polyandry in *M. celer* does not fit a “trading-up” strategy, with females remating only if the second male encountered is somehow superior to the first (e.g. Schneider & Elgar 1998). The decision to remate also seems independent of the female’s physical attributes or age. Similarly, virgin *M. celer* exhibit no obvious mate choice, passively accepting copulation from their first mate (Chelini & Hebets 2016). Together, results from virgin female matings (Chelini & Hebets 2016) and now second matings (present study) suggest that neither female nor male size (either cephalothorax width or leg length), or their interaction, are good predictors of copulation success. As such, the sexual size dimorphism observed in *M. celer* is unlikely to have been driven by mate-choice.

The apparent lack of benefits of polyandry could explain why we were unable to identify any decision criteria that females might be using to accept or reject a second mating. Our previous study focusing only on virgin females and their first mating similarly found no evidence of fitness benefits associated with male body size, leg length, or the degree of sexual size dimorphism between a female and her mate (Chelini & Hebets 2016). Variation in the benefits provided by males is often tied to the evolution of female mate choice (Hubbell & Johnson 1987; Kokko *et al.* 2003). As such, if fitness benefits were associated with *M. celer*’s male phenotype, we would expect it to be reflected in female mate choice decisions. The absence of mate choice could also indicate that polyandrous female *M. celer* are not trading-up, but rather bet-hedging, i.e., maximizing the genetic diversity of their offspring and minimizing the risk of infertility or low fitness (Watson 1991; Jennions & Petrie 2000; Fox & Rauter 2003). Benefits due to bet-hedging could be difficult or impossible to identify in a short term experiment (Holman 2015), which could explain the apparent lack of benefits of *M. celer*'s polyandry. Long-term field studies would be required to test the hypothesis that *M. celer* is bet-hedging and that the benefits of polyandry are not tied to the males' characteristics.

Protandric species such as *M. celer* are typically expected to have first male sperm priority, with the first male to mate with a virgin female fertilizing most of her eggs (Wiklund & Forsberg 1991; Zonneveld 1996; Kvarnemo & Simmons 2013). In spiders, the morphology of female reproductive tract is also hypothesized to influence the pattern of fertilization (Austad 1982). Specifically, entelegyne spiders such as *M. celer* have a "conduit" reproductive tract, where the first sperm entering the spermatheca is considered the most likely to be used in egg fertilizations (Elgar 1998). Nonetheless, many exceptions to this pattern have been described, and the most common sperm-precedence pattern in spiders seems to be sperm-mixing (see (Elgar 1998; Herberstein *et al.* 2011). Given that *M. celer* show both early male maturation and a "conduit" reproductive tract, we predicted *a priori* that this species presented first male sperm priority. Contradicting our prediction, our results suggest a strong degree of sperm mixing in first egg sacs of *M. celer* females.

Surprisingly, second males tended to fertilize most of the subsequent egg sacs, showing a putative advantage for males to mate with a previously mated female. The likelihood that a female *M. celer* may lay more than one egg sac in the field is likely highly dependent on the season‘s length and on the date of her first copulation, making it difficult to estimate the magnitude of this advantage under natural conditions. We do know, however, that female aggression increases following her first copulation, increasing the risk of sexual cannibalism (see results from Chelini & Hebets 2016 and present study). Thus, from a males' standpoint, the most advantageous strategy might be to benefit from the ready acceptance of virgin females to copulate, and guard these females after copulation until their short window of receptivity is closed. Although a few males have been observed guarding their mates in the laboratory and in the wild (Chelini & Hebets 2016, Chelini, *pers. obs*.), postcopulatory mate guarding does not seem to be a widespread strategy in this species.

With or without postcopulatory mate-guarding, the decision of *M. celer* females to remate may still be partly under male control. Some male spiders, and many insects, may influence a female's receptivity to subsequent matings through their seminal fluids (Ringo 1996; Aisenberg 2009; Wigby *et al.* 2009; Sirot, Wolfner & Wigby 2011). Substances incorporated into the seminal fluid may allow males to benefit from the access to virgin females gained through early maturation while minimizing the risks of decreased paternity by reducing females' likelihood to remate (Elgar & Bathgate, 1996; Elgar, 1998; Rice, 1996) As the number of mating partners does not seem to influence *M. celer* females' reproductive success (present study), a strategy of male-driven female monandry would not impose costs on females, while potentially conferring great benefits to males under scenarios of male-biased sex ratio and high male-male competition. The seminal fluid composition of *M. celer* and the relationship between seminal fluid transmission and female mating decisions are yet to be explored in depth.

Though mating strategies of females and males are hypothesized to be driven by their respective costs and benefits, these are often tested (perhaps simply for logistical reasons) in species where these costs and benefits are fairly evident (Huber 2005; Kvarnemo & Simmons 2013; Boulton & Shuker 2015). In this study, we present a species that defies many predictions regarding traditional mating systems. Species such as *M. celer*, provide an important challenge to our understanding of the coevolutionary dynamics of male and female mating strategies and the often assumed role played by pre and postcopulatory sexual selection on the evolution of sexual size dimorphism (Vollrath & Parker 1992).

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CHAPTER 3

Proximate mechanisms of sexual size dimorphism: integrating growth trajectories and metabolic rates

Sexual size dimorphism (SSD) is common in animals, but little is known about its underlying mechanisms. Organisms can only grow when the energy they ingest surpasses their energetic needs for maintenance and activity, and the time span of an organism's ontogeny should correlate with its adult size. In the crab-spider *Mecaphesa celer* (family: Thomisidae) females are approximately 1.5 times larger than males. We hypothesized that 1) females will grow faster than males; 2) females will have a lower mass-specific metabolic rate than males throughout their ontogeny and 3) female growth is more susceptible to the influence of diet and temperature than male growth. We show that *M. celer*'s SSD results from faster female growth over a longer period of time. Female and male metabolic rates did not differ, scaling with mass with a slope of 0.58. Diet by itself had no influence on male size but affected female size through an interactive effect with temperature: only females raised under a high diet with cool temperatures reached significantly larger sizes than females in the other treatments. We demonstrated that growth rate, and not simply number of instars, are the proximate determinants of the degree of SSD. Females may achieve a higher growth rate not by having lower maintenance metabolism than males, but through a combination of high food ingestion and low activity levels. Our results highlight the need for theoretical models linking the energetics of ontogenetic growth to variation in the fitness benefits of differing male and female sizes and behavior.

Introduction

Body size is one of the most important attributes of any organism, as it influences processes ranging from intracellular reactions to community stability (e.g. Gillooly *et al*. 2001; Savage *et al*. 2004; DeLong *et al.*, 2015). In species with sexual size dimorphism (SSD), body size and its associated processes vary widely between the sexes. Differences in the balance between selection for survival and selection for reproduction lead to differences in female and male sizes, and the fitness benefits associated to different body sizes have been studied in depth in a wide range of taxa (Blanckenhorn 2000; Blanckenhorn 2005; Cox & Calsbeek 2009; Preziosi & Fairbairn 2000). Understanding the evolution of SSD, however, depends not only on the identifying ultimate mechanisms generating fitness benefits for differences in size, but also how these organisms reach drastically different sizes at adulthood despite sharing a similar physiology and a majority of genes (Fairbairn 1997; Badyaev 2002a; Teder 2013; Chou *et al.* 2016).

SSD is typically underlined by differences in growth trajectories: sexes may be born with different sizes, one sex may grow for longer than the other, or one sex may grow faster than the other (Badyaev 2002b; Blanckenhorn *et al.* 2007; Stillwell *et al*. 2010; Teder 2013). Differences in egg size or size at birth are extremely rare in arthropods (Stillwell *et al.* 2010; Teder 2013; but see Budriene *et al.* 2013 for an example), but not in birds (see review by Weatherhead & Teather 1994) and mammals (e.g. sea lions: Ono & Boness 1996 and primates: Smith & Leigh 1998). The difference in size at birth is, however, seldom proportional, or even congruent, with the degree of SSD reached at adulthood (Badyaev 2002, Weatherhead & Teather 1994). Differences in growth length, growth rate, and a combination of both have been described in mammals (Leigh & Shea 1996; Badyaev 2002b and references therein, Garel *et al.*, 2006), reptiles (Shine 1994) and a vast number of arthropods (see reviews in Blanckenhorn *et al.* 2007, Stillwell & Davidowitz 2010 and Teder 2013).

Differences in growth rates between the sexes may decouple the relationship between development length and size at maturity, as both sexes may reach very different sizes in a similar time frame (Dmitriew 2011). Growth and development also may be influenced by environmental factors such as diet and temperature, such that differences in seasonal timing or geographic location of ontogeny between the sexes could lead to variation in each sex' growth and, consequently, in a species' degree of SSD (Badyaev 2002a; Stillwell & Davidowitz 2010). As such, in order to understand the expression of SSD in a given species, we must not only determine the developmental trajectories of females and males, but also understand the effects of the environment on these trajectories.

Understanding how growth trajectories underlie SSD raises an additional fundamental question: how does the larger sex fuel its faster growth? The energy assigned to growth depends on an individual's energy intake (foraging) and on the energy spent maintaining its existing tissues (i.e. its standard metabolic rate) and through activity (Hou *et al.* 2008; Dmitriew 2011). In species with SSD, we might therefore predict that the two sexes will reach different adult sizes by differing in their metabolic expenses (i.e., energy allocated to maintenance), or in the amount of energy they acquire from the environment. In other words, the larger sex should have a lower mass specific metabolic rate than the smaller sex (e.g. Ono & Boness 1996) or a higher food intake (see review by Shine 1989).

Spiders offer an ideal system in which to study the mechanisms underlying SSD as extreme degrees of SSD, mostly female-biased, have evolved multiple times within the group (Scharff & Coddington 1997; Hormiga *et al.* 2000; Legrand & Morse 2000). In addition, spiders are easy to raise, maintain and manipulate in the laboratory. Despite the abundance of studies focusing on the evolution of SSD in spiders (see references in *Chapter 1*), the proximate mechanisms underlying SSD are still mostly unknown. Here, we aim to understand the proximate mechanisms of SSD in the crab spider *Mecaphesa celer.*

Female *M. celer* are 1.3 to 2 times the body size of males, but often weigh more than 10 times the average male. Several crab-spiders species, including *M. celer*, are protandric (i.e., males mature earlier than females along the season – Muniappan & Chada 1970, Dodson & Beck 1993, Morse 2013a), so differences in development length may be least partially responsible for the SSD in this species. In previous studies of laboratory raised *M. celer*, differences in male and female developmental length correspond to two additional instars: males mature after six to seven molts, while females typically need 8 or 9 molts to reach maturity (Muniappan & Chada 1970). The degree of SSD reached at adulthood is not, however, proportional to the observed differences in growth length – females double in size in their last thee instars, but mature only a few weeks after males (Chelini, *pers. obs*). Our goals in this study are to: (1) determine the growth trajectories of female and male *M. celer*, testing the hypothesis that females grow faster than males; (2) determine how females and males differ in the energetics of their growth by testing the hypothesis that females fuel their faster and/or longer growth by having a lower standard metabolic rate than males; and finally, (3) determine the effect of diet and temperature on the growth of each sex.

Methods

Growth trajectories

To test the hypothesis that differences in growth trajectories underlie SSD in *M. celer*, we followed the growth of 250 *M. celer* spiderlings beginning at their second-instar (first-instar spiderlings are extremely small and fragile, and remain aggregated around the egg sac until their first molt). These spiderlings were born in the laboratory between September and October 2014. They were the offspring of 48 females that were collected in Lincoln, NE, USA in May 2014 and mated in the laboratory in August 2014. We randomly selected 5-10 spiderlings from each clutch and housed them in individual 4 x 4 x 6 cm acrylic cages, with *ad libitum* water, and small pieces of netting for perching, in a room at 26° C and 60% relative humidity, under a 14:10 light:dark cycle. We checked all spiderlings for molts three times a week. After each molt, we photographed each spiderling with a Spot Flex® digital camera (Model 15.2 64 MP, Diagnostic Instruments, Inc.) mounted on a Leica® DM 4000 B Microscope. We fed all individuals twice a week with flightless *Drosophila melanogaster* in quantities corresponding to each spiderling's age: third instar spiderlings were fed three *D. melanogaster*, fourth instar spiderlings were fed four *D. melanogaster*, etc., We selected a subset of 30 female and 30 male spiderlings that successfully reached adulthood for size and growth quantifications. As spiderling sex can only be identified at their fifth instar, our sample size was limited by the high mortality rates of early instars spiderlings, whose sex was unknown. We used only one male and one female spiderling per family to prevent confounding genetic effects.

We measured the cephalothorax of each spiderling at each of their developmental instars from the photographs taken after their molts with ImageJ

(Rasband 1997-2012). We calculated the age of the spiderlings in days after birth and kept track of the date (+/- 2 days) and number of their molts, which allowed us to determine the average length, in days and in instars, of each sex's growth.

We tested for differences in female and male growth trajectories using two Gamma Generalized Linear Mixed Models (GLMMs) with a log link. In one model, we used the size of each spiderling at each instar as the response variable, instar and sex as predictor variables, and the spiderling ID as a random effect. In a second model, we replaced the instar by their age in days. We ran these analyses using the function *glmer* in R from the package *lme4* (R Development Core Team 2009, Bates *et al.* 2015).

Energetics of growth: Metabolic rate measurements

To test the hypothesis that males have a lower mass-specific metabolic rate than females throughout their ontogeny, we measured oxygen consumption rate of 146 spiderlings described above using an OXY-10 micro fluorescent oxygen sensor (PreSens, Regensburg, Germany). To ensure that oxygen consumption measurements were for resting spiders, we placed each spiderling at the bottom of a 0.5 ml Eppendorf® microcentrifuge tube and held them in place by a circular 2.5 mm diameter piece of netting (allowing oxygen flow but reducing spiderling movement). We then placed these microcentrifuge tubes in a thermocycler at 26° C, where spiderlings acclimated for 30 minutes before we started recording their oxygen consumption. We calculated the metabolic rate of each spiderling as the slope of the linear regression between mass of oxygen inside the Eppendorf® tubes against time (mg O_2/min). We used only the shallowest segment of each spiderling's oxygen consumption slope for our estimate of standard metabolic rate to avoid including periods of spiderling stress and for consistency across measurement. To avoid quantifying metabolic costs of digestion and molting, we controlled the timing of our measurements: we measured metabolic rates two days after a feeding day and between one to three weeks following their previous molt (instars vary in length from five to 80 days - Muniappan & Chada 1970). We randomly selected 10-20 individuals of each sex to be tested at each instar, with no repeated measures.

We weighed each spiderling to the nearest 0.1 mg immediately after measuring their metabolic rates using an Ohaus® precision scale. Data on oxygen consumption rate and mass were log transformed to linearize the relationship between metabolic rates and size/mass. We used a linear mixed model (LMM) to determine the relationship between standard energetic demands and sexual size dimorphism, using the natural log of the metabolic rate as our response variable, sex, natural log of mass and their interaction as predictor variables, and spiderling family as a random effect. We then conducted a likelihood ratio test between this full model, a model with log of mass as the sole predictor variable, and a null model. We ran these analyses using the function *lmer* from R's package *lme4* and the function *anova* (R Development Core Team 2009, Bates *et al.* 2015).

Environmental effects on SSD: Diet and temperature manipulation

To determine the effects of diet and temperature on the degree of SSD of *M. celer*, we subjected 1000 spiderlings to four treatments in a 2 x 2 factorial design involving two diet treatments (High diet versus Low diet) and two temperature treatments (Warm versus Cold) (Table 1). All spiderlings were born in the laboratory, resulting from the same mating trials described in *Growth trajectories* (above). Siblings were equally distributed among all treatments. The Warm treatment was identical to the environmental conditions described above (see *Growth trajectories*). Cold treatment spiderlings were reared under temperatures that simulated mild seasons of the year, decreasing progressively from 26° C (September - October) to 18° C (November - December) and to 13° C (January - April), then increasing again to 16° C (May - June), 18° C (July - August) and finally 20° C, with mild fluctuations in temperature (+/- 5° C) occurring during each of the "seasons" and lasting from one to three days. We used a 14:10 light:dark cycle for all treatments. High-diet spiderlings were fed with flightless *D. melanogaster* according to the schedule described in *Growth trajectories*: in quantities corresponding to each spiderling age, twice a week. Low-diet spiderlings were fed the same diet, but only once a week. The spiderlings used in our analyses of *Growth trajectories* and *Energetics of growth* were the same as those of our High diet – Warm treatment. Mature spiders were then photographed and measured following the same methods used for spiderlings (see *Growth trajectories* for details).

Table 1: Summary of the diet and temperature treatments used to test the effects of the environment on the sexual size dimorphism of *Mecaphesa celer*.

High diet Warm (HW) 26° C, *D. melanogaster* 2x per week n = 250	**Low diet Warm (LW)** 26° C, *D. melanogaster* 1x per week n = 250
High diet Cold (HC) 13° C to 21° C, *D. melanogaster* 2x per week n = 250	**Low diet Cold (LC)** 13° C to 21° C, *D. melanogaster* 1x per week n = 250

We tested for an effect of diet and temperature on the degree of SSD in *M. celer* using separate linear models (LM) for females and males. We used diet treatment, temperature, and their interaction as predictor variables of the size of each sex at adulthood. Upon finding significant effects of one or two of the predictor variables, we ran Tukey post-hoc tests to identify differences between the treatments. We ran these analyses using the function *lme* from R's package *lme4* (R Development Core Team 2009, Bates *et al.* 2015) and the function *glht* from the package *multcomp* (Torsten *et al.* 2008).

Results

Growth trajectories

Female and male *M. celer* showed different growth trajectories (Fig 1). Females matured in 9 to 11 instars, while males matured in 6 or 7 instars. Females on a High diet and on constant Warm temperatures (HW) reached maturity in an average of 273.7 days (min = 102, max = 394), while males took on average 204 days (min = 109, max = 345). Females and males differed significantly in the size achieved in each instar (Table 1), as well as in the relationship between size and age in days (Table 2).

Table 1: Gamma GLMM testing the relation between *Mecaphesa celer* size at each developmental instar and sex. N = 304, Deviance = -520.7, $\chi^2 = 844.9$, d.f = 3, $p < 0.001$.

	Estimate	St. Error	*t*	P
Intercept	-0.57	0.03	-22.04	<0.001
Instar	0.16	0.03	61.28	<0.01
Sex	0.10	0.04	2.59	0.009
Instar * Sex	-0.03	0.005	-6.01	<0.001

Table 2: Gamma GLMM testing the relation between *Mecaphesa celer* size, age in days and sex. N = 304, Deviance = -216.19, $\chi^2 = 540.34$, d.f = 3, $p < 0.001$.

	Estimate	**St. Error**	***t***	**P**
Intercept	0.45	0.05	-9.16	<0.001
Age (days)	0.005	0.0001	35.63	<0.001
Sex	0.13	0.07	1.84	0.07
Age * Sex	0.002	0.0002	-6.45	<0.001

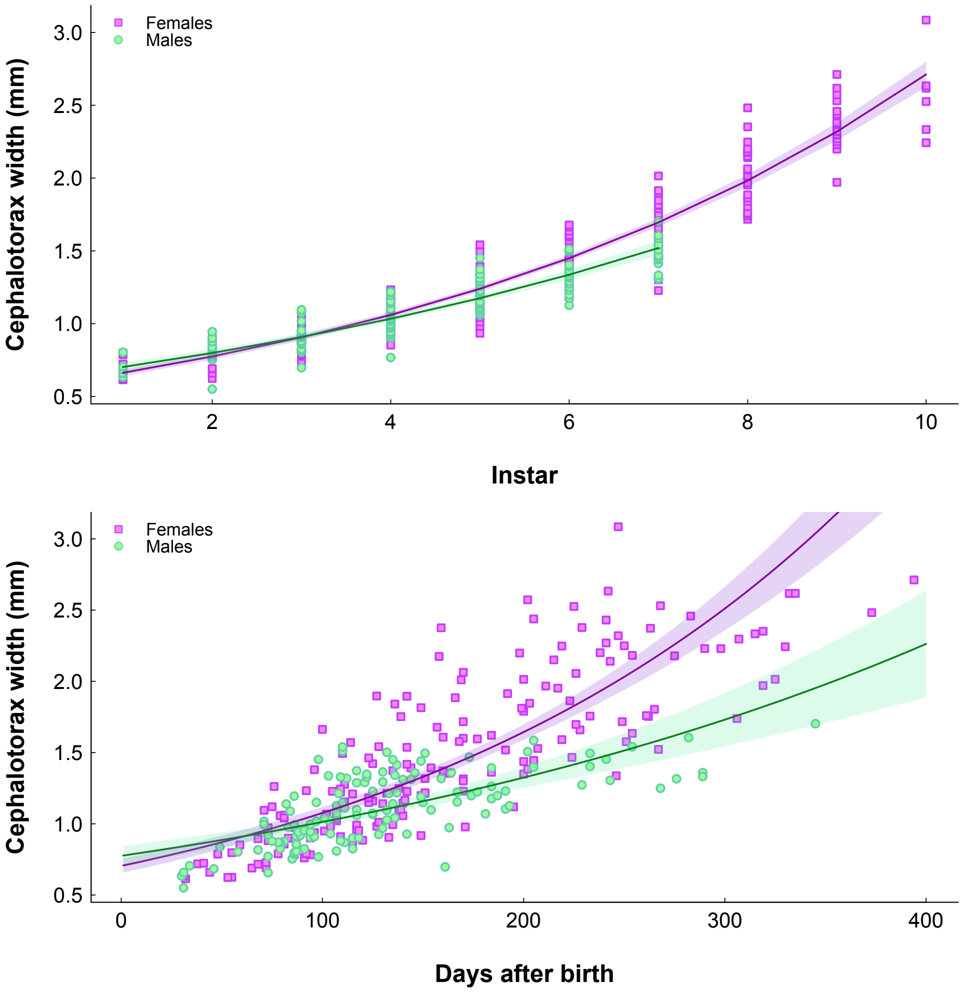

Fig. 1: Growth trajectories of female and male *Mecaphesa celer*. Females have up to five developmental instars more than males, but also grow faster.

Energetic demand quantification: Metabolic rate measurements

M. celer's metabolic rate scaled with mass with a slope of 0.58. This slope did not differ between females and males (Likelihood ratio test of models with and without the variable "sex": χ^2 = 1.33, p = 0.51, Table 3, Fig. 2).

Table 3: LMM testing the effect of log of mass (mg), sex and their interaction on the metabolic rate (mg O_2 consumed/min) of *Mecaphesa celer* spiders along their ontogeny. Spiderling families were used as random effect. χ^2 = 43.24, d.f. = 3, p = 2.19e-09.

	Estimate	**St. Error**	***t***
Intercept	-13.63	0.22	-62.74
Sex	-0.05	0.46	-0.12
Log Mass	0.49	0.09	5.36
Sex * Log Mass	-0.07	0.28	-0.24

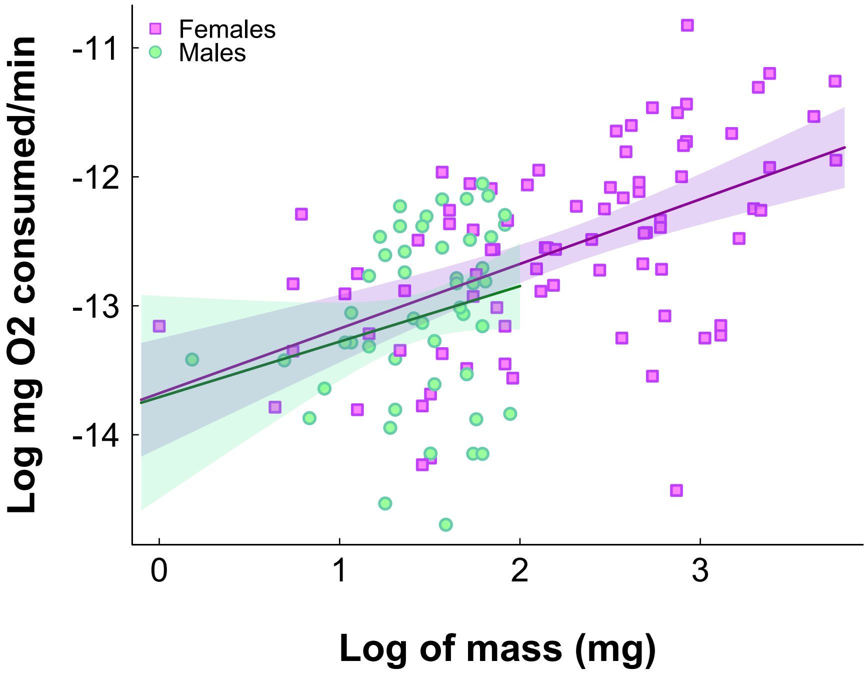

Figure 3: Metabolic rate scaling with mass of both female and male *Mecaphesa celer* at different points of their ontogeny.

Environmental effects on SSD: Diet and temperature manipulation

Diet, temperature and their interaction affected *M. celer* females' size (Table 4). Females in the High-diet – Cold treatment were the only ones significantly different from those of all the other treatments (Table 5, Figure 4). Male size was not affected by diet, temperature or their interaction. (Table 6, Fig. 4).

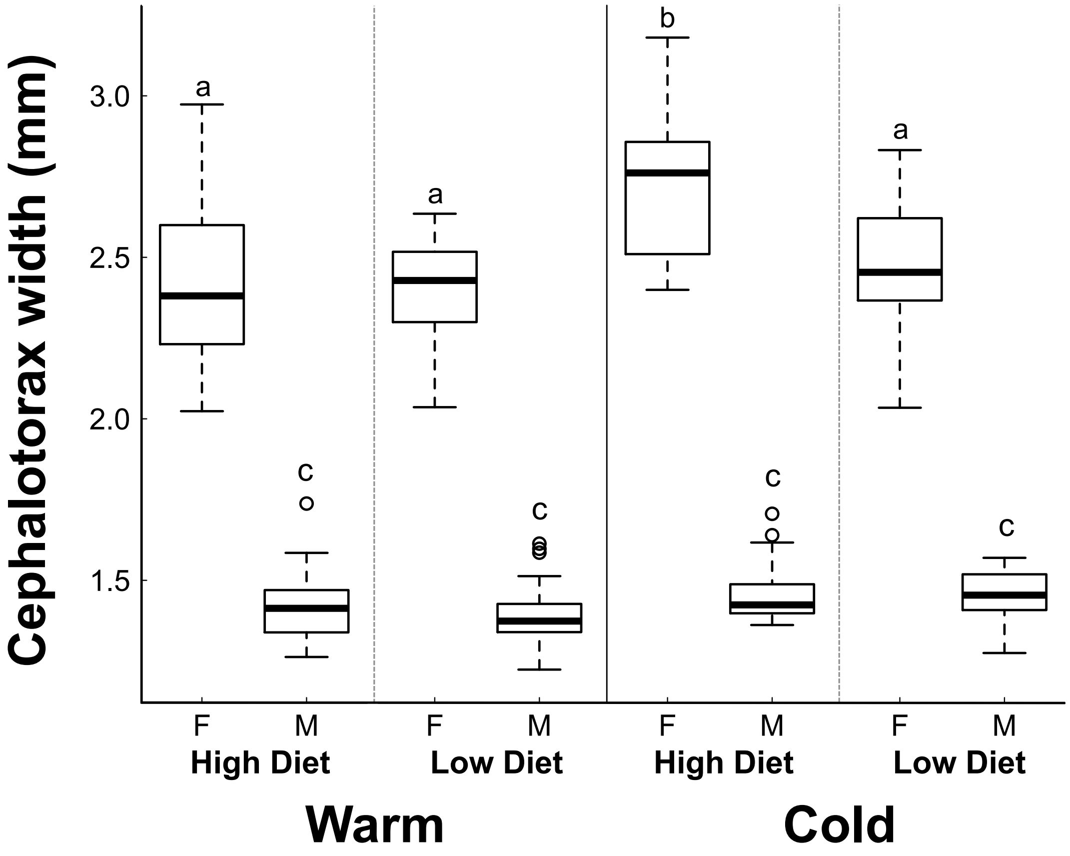

Fig. 4: Cephalotorax width of adult female and male *Mecaphesa celer* in two diet and temperature treatments. Boxes correspond to 1st quartile, median and 3rd quartile, whiskers correspond to the range. Within a sex, boxes with different letters are significantly different from each other.

Table 4: Linear model testing the effect of diet, temperature and their interaction on the size (cephalotorax width) of adult female *Mecaphesa celer* spiders. Multiple R-squared: 0.2066, R-squared: 0.185, F = 9.55, d.f = 110, $p < 0.001$

	Estimate	**St. Error**	***t***	**P**
Intercept	2.71	0.05	52.21	<0.001
Diet	0.24	0.07	-3.65	0.0004
Temperature	-0.31	0.06	-5.04	<0.001
Diet * Temperature	0.24	0.08	2.88	0.005

Table 5: Tukey post-hoc comparisons between the size (cephalotorax width) of adult female *Mecaphesa celer* spiders in different combinations of diet and temperature.

Treatments	Estimate	St. Error	*t*	P
High Warm – High Cold	-0.31	0.06	-5.04	< 0.0001
Low Cold – High Cold	-0.24	0.06	-3.65	0.002
Low Warm - High Cold	0.31	0.06	-4.73	< 0.0001
Low Cold - High Warm	0.07	0.05	1.32	0.55
Low Warm- High Warm	0.001	0.05	-0.03	0.99
Low Warm - Low Cold	0.07	0.06	-1.23	0.61

Table 6: Linear model testing the effect of diet, temperature and their interaction on the size (cephalotorax width) of adult male *Mecaphesa celer* spiders. Multiple R-squared: 0.07, Adjusted R-squared: 0.04, F = 2.74, d.f. = 116, $p = 0.046$

	Estimate	**St. Error**	***t***	**P**
Intercept	1.46	0.02	65.46	<0.001
Diet	-0.01	0.03	-0.46	0.65
Temperature	-0.03	0.03	-0.96	0.34
Diet * Temperature	-0.03	0.04	-0.80	0.43

Discussion

Our results indicate that SSD in *M. celer* results from a combination of additional instars and faster female growth. Surprisingly, this longer and faster growth does not seem to be fueled by lower resting metabolism, which would allow females to allocate more energy to growth and less to body maintenance. On the contrary, female and male metabolic rates scaled with mass according to the same slope of 0.58. Also surprisingly, diet had little to no influence on the size obtained by females and males at adulthood. Only females raised under a High diet with Cold temperatures following a seasonal pattern reached significantly larger sizes than females raised in the same temperature treatment but with a Low diet, or those raised in a constantly Warm environment. This interactive effect between diet and temperature on female size suggests that seasonality may play an important role in the magnitude of SSD. Such an effect may contribute to intraspecific variation in degree of SSD observed in many animal species that experience strong seasonality.

Our results indicate faster growth rates for females versus males, but it is still unclear how this faster growth rate is fueled. Although differing in many aspects, most models of ontogenetic growth predict a relationship between growth rate, the amount of energy ingested, and the cost of maintaining an organism’s existing tissues (e.g. West *et al.* 2001; Ricklefs 2003; Moses *et al.* 2008; DeLong 2012). Our observed differences in growth rate could then be due to two main mechanisms: (i) different energetic supply (i.e. energy ingestion) and/or (ii) differences in energetic demand (i.e. in the amount of energy spent by each sex) (Dmitriew 2011). We did not find any evidence that differences in resting metabolism underlie SSD in *M. celer*. Resting metabolic rate represents, however, only a fraction of the energy spent by an organism: males and females may also differ in metabolic costs other than the maintenance of their existing tissues, or may differ in the energy spent through their activities.

Sex-specific growth rates in *M. celer* could be related to differences in each sex’ active metabolic rate (i.e., metabolic rates reached during activity) rather than standard metabolic rate (e.g. Downs *et al.* 2016). Differences in the metabolism during exercise have been reported for sexually dimorphic arthropods (e.g. Rogowitz & Chappell 2000; Gäde 2002), but so far such differences have not been observed in spiders (Shillington & Peterson 2002). It is worth mentioning that very few studies on spiders have measured metabolic rates of both sexes while controlling for foraging state and reproductive status (two factors known to greatly influence standard metabolic rate – Burton *et al.* 2011). Those studies found contrasting results, with females or males exhibiting higher metabolic rates, or with no difference being found between the sexes (Kotiaho 1998; Walker & Irwin 2006).

Even if metabolic rates during activity are the same for both sexes, females and males may also differ in their energetic expenditure simply through differences in behavior. In multiple sexually size dimorphic species females and males differ in their activity levels and overall life-history (Vollrath & Parker 1992; Vollrath 1998; Beck *et al.* 2007). The best known examples of extreme SSD are spiders whose females are highly specialized sit-and-wait predators, while males typically spend their adult instar roaming in search of females (Walker & Rypstra 2001, 2002; Foellmer & Moya-Laraño 2007; Dodson *et al.* 2015). An analysis of female and male activity levels throughout their ontogeny would be required to test this hypothesis in *M. celer*.

Alternatively, or in addition to differences in activity levels, SSD may be proximately caused by differences in the energetic supply for each sex. The energy spent searching for prey or foraging patches, as well as capturing each prey item, increases with body size following a cubic slope (Tenhumberg *et al.* 2000). Regardless of how much food is available to them in the wild, small sized organisms may benefit by minimizing the foraging effort necessary to reach satiation, allowing more time and energy to be spent in reproductive activities (Blanckenhorn *et al.* 1995;

Blanckenhorn & Viele 1999; Blanckenhorn 2005). In species where there are no benefits associated with being large (such as previously suggested for *M. celer*, see Chelini & Hebets 2016a, Chelini & Hebets 2016b), optimal male size would therefore be selected for by the time and energy benefits of being small, and would remain relatively fixed regardless of the foraging conditions under which males grow (Shine 1989; Blanckenhorn 2000). Females, on the other hand, are likely to be under selection for larger sizes and increased food consumption due to the fecundity benefits associated with size (Shine 1988; Honek 1993; Preziosi *et al.* 1996). We can therefore hypothesize that female biased SSD in sit-and-wait predators such as crab spiders or orb-weavers is proximately caused by a combination of female higher food intake and lower energetic demands due to lower activity. According to this hypothesis, in our experiment, diet did indeed have an effect on female size, but not on male size.

Only females in the High diet – Cold treatment reached significantly larger sizes than those in the other treatments. The interaction between diet and temperature on female *M. celer* body size is in accordance with the temperature-size rule, which predicts that ectotherms will develop for longer and reach larger sizes at lower temperatures (Atkinson 1994). As metabolic rates decrease with temperature (Gillooly *et al.* 2001; see Schmalhofer 2011 for an empirical example with crab-spiders), females from the Cold treatment could invest more of their energetic supply towards growth than those of Warm treatments. However, growth is known to be plastic in many arthropods, varying according to temperature, diet, days between feeding events, photoperiod, among others (see review by Stillwell *et al.* 2010; Dmitriew 2011, but see Hirst *et al.*, 2015). As such, the lack of difference between High diet – Warm, Low diet – Warm and Low diet – Cold females is likely due to a complex interactive effect of energetic supply and temperature on standard metabolic rates and growth.

Our results add to the significant body of evidence indicating that SSD is proximately caused by differences in both the growth length and the growth rates of females and males (Badyaev 2002a; Esperk *et al.* 2007). As in many other arthropod species, female *M. celer* achieve a body size of nearly twice the size of males by undergoing additional molts, but we demonstrated here that growth rate, and not simply number of instars, are the determinants of the degree of SSD. Differences in standard metabolism do not underlie SSD in this species, suggesting that females achieve a higher growth rate through a combination of high food ingestion and low activity levels. Moreover, our results highlight the need for theoretical models of ontogenetic growth that incorporate intraspecific variation when predicting the relations between energetic supply, standard metabolic rates and growth. As we gain a better understanding of the growth trajectories leading to SSD, we must take into account ontogenetic differences between the sexes and develop new theoretical models predicting the relation between metabolism and growth in order to fully understand the evolution of SSD.

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CHAPTER 4

Field evidence challenges the relationship between early male maturation and female-biased sexual size dimorphism

In many species in which females are larger than males (female biased sexual size dimorphism -SSD) males mature prior to females (i.e. protandry). Protandry may benefit males because it facilitates access to virgin females, particularly in monogamous species, and early maturation may indirectly lead to SSD due to a reduction of males' development time. In contrast, selection may act directly on SSD through selection for large female size or small male size, with protandry as an indirect consequence. We use field data collected along the entire reproductive season of a crab spider, *Mecaphesa celer*, to test three predictions of the mating opportunity hypothesis: 1) males mature prior to females in the field; 2) the proportion of virgin females decreases rapidly along the season, so early-matured males have an advantage over late-matured males; and 3) early-matured males will be smaller than late-matured males, indicating a relationship between early male maturation and female biased SSD. Maturation times of males and females collected throughout the season show a clear pattern of protandry. The proportion of virgin females decreased rapidly in the first weeks of the season, then increased towards the end of the season. Size measurements indicate a pattern in which both sexes' size peak before the middle of the reproductive season, and then decrease. Our results provide partial support for the mating opportunity hypothesis. SSD seems to be a consequence of protandry in this species, but the high plasticity in female and male growth rates challenges the assumption that SSD in spiders is only a consequence of selection for early male maturation.

Introduction

Notable differences in size between females and males of a species, or sexual size dimorphism (SSD), are typically attributed to selection for survival and reproduction balancing out differently for each sex (SSD equilibrium model: Blackenhorn 2000; Blackenhorn 2005). The evolution of male-biased SSD, i.e. males being larger on average than females, is common in species with intense direct male-male competition (see Fairbairn 1997 and references therein), while female-biased SSD, where females are larger on average than males, is expected to evolve due to the fecundity benefits of large female size (Shine 1988, 1989; Preziosi *et al.* 1996).

Across taxa, female-biased sexual size dimorphism is often associated with protandry, or males reaching sexual maturity before females (Blanckenhorn 2000). In protandric species, early maturation should facilitate male access to virgin females and is particularly important in monogamous species or species with first male sperm priority (Wiklund & Fagerström 1977; Fagerström & Wiklund 1982; Blanckenhorn *et al.* 2007). Protandry may, however, also lead to intense competition for mates: strongly male-biased operational sex ratios early in the reproductive season lead to high levels of male-male competition that should slowly decrease as more females mature and the sex ratio becomes more equitable or even female biased (Vollrath & Parker 1992; Legrand & Morse 2000). The benefits of protandry therefore vary greatly with the synchrony of female maturation: if all females mature in a short window of time, males benefit by maturing earlier. If female maturation is spread along the season, males may find virgin females at any moment, decreasing the benefits of early male maturation. As such, the male fitness benefits of protandry depend on the timing of their own maturation relative to the females' and relative to the maturation of other males (Holzapfel & Bradshaw 2002; Kasumovic & Andrade 2009).

A strong relationship between protandry and female-biased sexual size dimorphism has often been predicted theoretically, (e.g. Wiklund & Fagerström 1977; Abrams *et al.* 1996; Alcock 1997; Morbey 2013) and observed empirically (e.g. Alcock 1997; Stillwell *et al.* 2010; Smith & Brockmann 2014). Multiple hypotheses have also been proposed to explain the evolution of protandry (see review by Morbey & Ydenberg 2001), and two of these relate directly to the evolutionary relationship between protandry and SSD. First, the constraint hypothesis predicts that protandry is a by-product of selection for another trait, such as large female size (e.g. Matsuura 2006). According to this hypothesis, SSD and protandry would only evolve together if females' and males' growth rates are fixed, i.e., not plastic. Selection for large female body size (and/or small male body size) would therefore require longer female growth. Second, the mating opportunity hypothesis suggests that early male maturation is directly selected for, and female-biased SSD evolves as a by-product of such selection. Comparing these two hypotheses, female-biased SSD may be considered either a cause or a consequence of protandry.

Spiders are renowned for their frequent female-biased SSD (Prenter, Elwood & Montgomery 1998; Wilder, Rypstra & Elgar 2009), with some of the most extreme examples being found in orb-weaver (Araneidae) (Hormiga *et al.* 2000) and crab spiders (Thomisidae) (e.g. Chelini & Hebets 2016). As many species of crab spiders have low remating rates and exhibit no mate choice (Morse 2007a; Chelini & Hebets 2016), selection for a shorter development and scramble competition for virgin females are assumed to drive SSD in this family (Dodson & Beck 1993; Legrand & Morse 2000; Morse 2013a). In other words, the mating opportunity hypothesis is commonly invoked to explain the female biased SSD in crab spiders, as SSD is presumed to be a consequence of selection for early male maturation. In this study we use detailed field observations through the season to explore the relationship between protandry and SSD in a flower-dwelling species of crab spider, *Mecaphesa celer*.

Previous results from laboratory rearing and behavioral experiments provide mixed support for the mating opportunity hypothesis in *M. celer*. Females of this species are 1.5 to 2 times the size of males and may weigh up to 10 times the males' mass. In the laboratory, female *M. celer* have two to four developmental instars more than males, corresponding to an average difference of 70 days between male maturation and female maturation (Chelini *et al. Chapter 3*). Prior studies have found that female *M. celer* are only receptive to remating during a short window of time, with remating rates decreasing from 85 % to 15 % over two days after their first copulation (Chelini & Hebets 2016 *in press*). As the benefits of protandry are tightly associated with female monogamy, such results support the hypothesis that protandry is directly selected for in this species. The same study, however, also found that when females do mate with multiple males, second males may have a slight advantage in terms of sperm priority (Chelini & Hebets 2016 *in press*), which contradicts the mating opportunity hypothesis. Nothing is currently known about the degree of protandry or on the intensity of male-male competition in the field.

In this study, we use field data collected along an entire reproductive season to test three prediction of the mating opportunity hypothesis in *M. celer*. If SSD in *M. celer* is a consequence of selection for adaptive protandry (i.e. mating opportunity hypothesis), then we predict: 1) males mature prior to females in the field; 2) the proportion of virgin females decreases rapidly along the season, so late-matured males have fewer mating opportunities than early-matures males; and 3) early maturation leads to a reduction in male size, so early-matured males should be smaller than late-matured males.

Methods

Field observations

We followed a population of *Mecaphesa celer* from a 20 000 m^2 tall grass prairie patch at Holmes Lake park, Lincoln – NE, USA in 20 bi-weekly surveys between May 13th and July 31th 2015. Female and male *M. celer* are typically found on top of flowers during the warmest hours of the day. During each field survey we sampled all plants bearing flowers with beat sheets and sweeping nets, starting at 12:30 h. We aimed to collect at least 80 spiders per survey, or as many as we could get in four hours of collecting effort. For all *M. celer* individuals we found, we recorded their approximate age (based on their size), developmental status (mature/not mature), and sex (female, male or unknown, as sexual dimorphism becomes apparent only after the fifth instar). To obtain accurate measurements of size, we placed each individual in flat 2 x 2 cm sealable plastic bags and photographed them against millimeter graph paper. At the end of each survey trip we released all spiders in the general area and on the flower type of their original collection.

To estimate the likelihood of encountering a virgin female along the season, on the last survey of each week we randomly selected five to eight adult females to bring to the laboratory and be allowed to lay egg sacs. We calculated the proportion of females collected each week that laid fertilized egg sacs in the laboratory, and used it as a proxy for the proportion of females that were already mated in the field each week. In the laboratory, we housed these females individually in 4 x 4 x 6 cm acrylic cages in a room at 26° C and 60% relative humidity, under a 14:10 light:dark cycle. We provided them with *ad libitum* water and small pieces of netting for perching. We fed field collected females twice a week with two juvenile crickets (*Acheta domesticus*, 1 mm, Ghann's Cricket Farms – GA, USA), and monitored them every two days to check for egg sacs. Once females laid their egg sacs we stopped feeding them until the spiderlings had hatched and dispersed (females guarding egg sacs will not eat, and crickets may prey upon eggs – Chelini *pers. obs*.). Upon spiderling dispersion (3-5 days after egg sac hatching) we separated them from the mother, counted them, and sacrificed them by freezing. We returned the mothers to their cages and to their normal feeding schedule until they laid another egg sac, or until their natural death. We sacrificed all remaining females by freezing on the 18th of December 2015, after temperatures in the field had dropped below freezing.

To determine the relationship between timing of male maturation and degree of SSD we measured all adult individuals found during each survey. We measured each female's and male's cephalothorax width (the most appropriate measure of body size in spiders with SSD - Foellmer & Moya-Laraño 2007) on the photographs taken in the field using the software Image J (Rasband 1997-2012).

Statistical analyses

Prediction 1 - *Males mature prior to females in the field*

We tested whether males do indeed mature earlier than females in the field, with a binomial Generalized Linear Model (GLM), using the proportion of adult individuals as a response variable and the individuals' sex, the Julian date of each survey, and their interaction as predictor variables.

Prediction 2 – *The proportion of virgin females decreases rapidly along the season*

We tested whether the proportion of virgin females decreases along the reproductive season with a binomial GLM with the proportion of females brought to the laboratory that laid an egg sac as the response variable and the Julian date as the predictor variable. To combine these laboratory results with our field observations, we multiplied the proportion of pregnant females in the laboratory by the number of females found in the field, obtaining a rough estimation of how many females could already be mated at any given time in the field. The analyses of Predictions 1 and 2 were conducted with the functions *glm* from R's package *lme4* (R Development Core Team 2009, Bates *et al.* 2015).

Prediction 3 - *Early matured males are smaller than late-matured males*

We tested the relationship between male size and timing of maturation in *M. celer* with two Generalized Additive Models (GAMs), one for females and one for males, using the adult individuals' size as a response variable and a smooth function of the Julian date as a predictor variable. If SSD is a by-product of protandry, we expect male size to increase along the season (with early matured males being smaller than late matured males). These analyses were conducted with the function *gam* from R's package *mgcv* (R Development Core Team 2009, Wood 2011).

Results

Prediction 1 - *Males mature prior to females in the field*

We sampled a total of 1340 juvenile and adult *M. celer* throughout the season. In each field survey we collected between 96 and 37 individuals, with numbers declining abruptly from mid to late July (late season). Male *M. celer* mature significantly earlier than females in the wild (Table 1, Fig. 1, Fig. 2.). The operational sex ratio was male-biased throughout most of the season, with the exception of two weeks in which females were the most abundant sex (Fig. 1B).

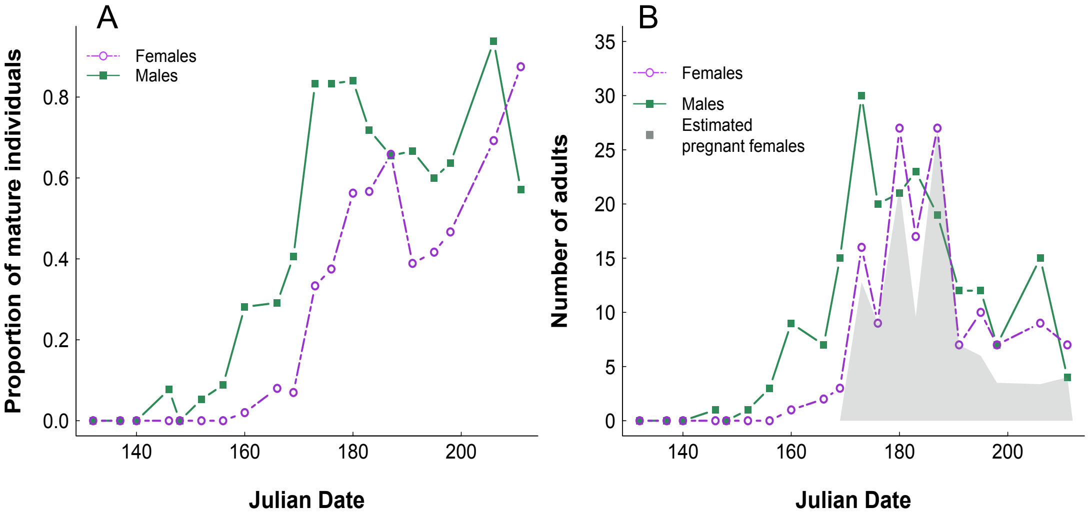

Figure 1: (A) Proportion of mature *Mecaphesa celer* individuals of each sex found along the reproductive season (May 13th to July 31st 2015). (B) Number of adult females and males per sex along the season. The grey area corresponds to the estimated number of mated females, based on the proportion of pregnant females collected in each field trip.

Table 1: Binomial GLM model on the probability of being mature according to sex and time along the season. Residual deviance = 1355.31, d.f. = 3, Deviance = -449.81, p < 2.2e-16.

	Estimate	**St. Error**	**z**	**p**
(Intercept)	-18.70	1.58	-11.82	<2.00E-16
Sex	6.35	2.12	2.99	0.002
Julian date	0.10	0.01	11.35	<2.00E-16
Sex * Julian date	-0.03	0.01	-2.40	0.02

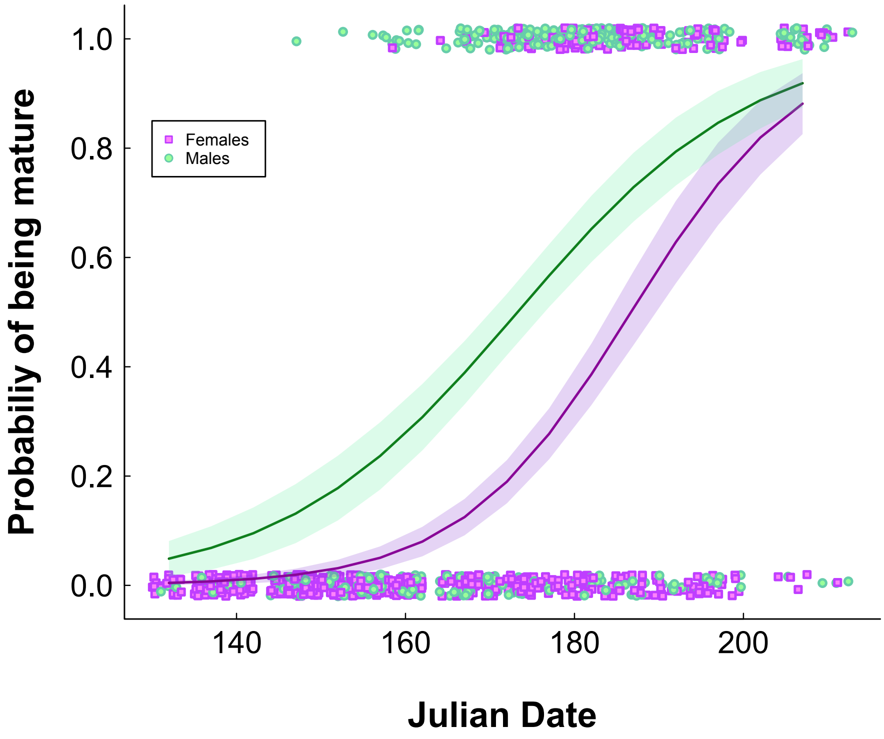

Fig. 2: Probability of finding mature female and male *Mecaphesa celer* in the wild along the reproductive season. Lines indicate the probability slope predicted by a binomial GLM and the shaded areas correspond to the 95% confidence intervals.

Prediction 2 - *The proportion of virgin females decreases rapidly along the season*

All *M. celer* females start the season as virgins, given that this species live for only one year. The proportion of females that did not lay egg sacs in the laboratory (i.e., virgin females) was very low early in the season, indicating that females do become rapidly mated, and increased significantly until late July (Table 2, Fig. 3). The highest proportion of virgin females in the field (i.e., lowest proportion of pregnant females) corresponds to the end of the reproductive season (Fig. 3).

Table 2: Binomial GLM model on the proportion of virgin females along the season. The proportion of virgin females increases with time. Residual deviance = 69.46, d.f. = 1, Deviance = 6.38, p < 0.01.

	Estimate	**St. error**	**z**	**P**
(Intercept)	12.9669	5.13515	2.525	0.011
Julian date	-0.0626	0.02627	-2.383	0.017

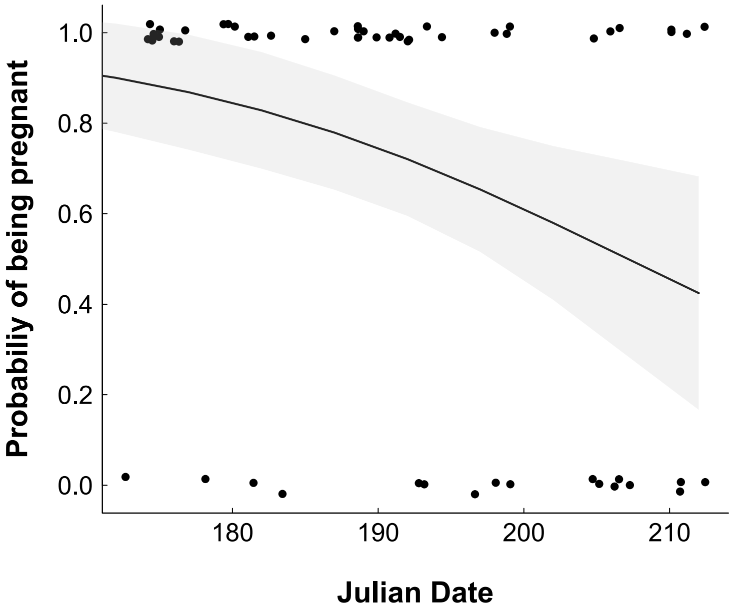

Fig. 3: Probability of females being pregnant along the reproductive season. The solid line represents the probability as predicted by the binomial GLM, and the grey area represents the 95% confidence interval.

Prediction 3 – *Early-matured males are smaller than late-matured males*

Across the 338 adult female and male *M. celer* individuals that we measured throughout the season, female and male size peaked in mid to late June, then decreased from late June to late August (Males GAM: F = 14.76, p = 3.06e-09, deviance explained = 21.7%; Females GAM: F = 8.25, p = 7.07e-05, deviance explained = 15.4%). The degree of SSD (average female/average male size ratio) remained similar throughout the season, varying from 1.48 in mid June to 1.66 in late August. (Fig. 4)

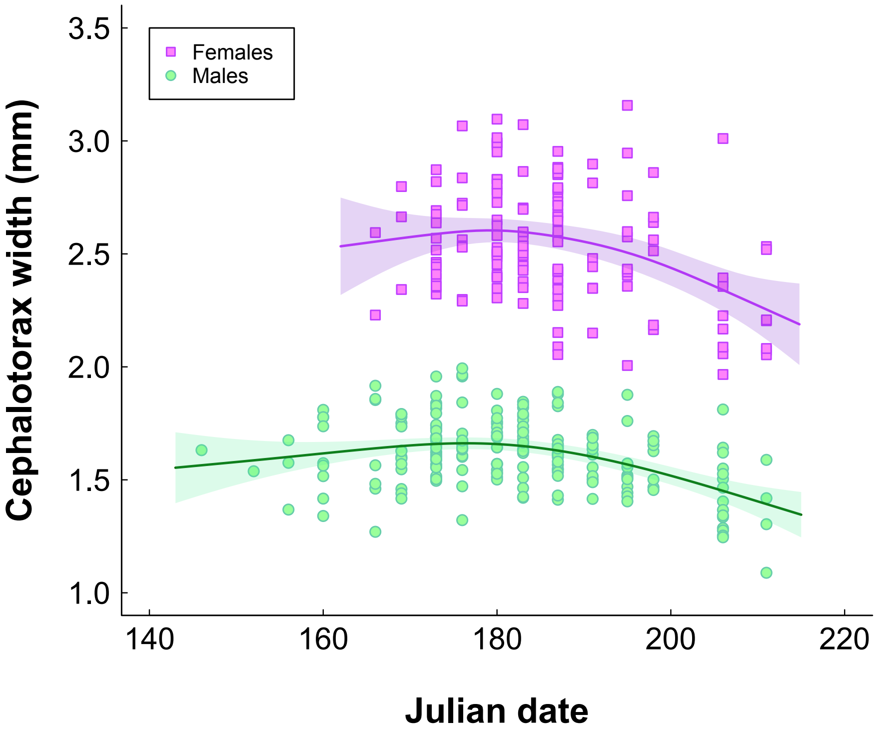

Fig. 4: Female and male *Mecaphesa celer* size throughout the season. Lines represent the values predicted by a GAM. Purple and green shaded areas represent female and male 95% confidence intervals, respectively.

Discussion

Field data collected throughout the season on a population of the female-biased SSD crab spiders *Mecaphesa celer* demonstrate that this species is clearly protandric, as males mature significantly earlier than females. Nearly all females became immediately pregnant after maturation in the first weeks of the season, indicating an advantage of early-male maturation. Surprisingly, however, close to half of the available females remain unmated in the last weeks of the season. As such, the proportion of virgin females decreased abruptly in the first weeks of the season, then increased towards the end of the season. Size measurements indicate a pattern in which the largest individuals of both sexes mature in mid to late June (mid season), after which females and males mature at progressively smaller sizes until the end of the season.

The difference in maturation times between females and males is much shorter in the field that what had previously been reported in laboratory experiments (Chelini *et al. Chapter 3*). Nonetheless, males matured earlier than females, with close to 90% of males being mature one week prior to the peak of female maturation, supporting our first prediction. Our seasonal size and development data provide no support for the constraint hypothesis, which poses that selection for large female size selects indirectly for early male maturation due to fixed growth rates of females and male. Indeed, the discrepancy between degree of protandry previously reported in the laboratory (Chelini et. al, *Chapter 3*) and the degree of protandry seen in the field indicates that female and male growth trajectories are not fixed, but rather very plastic.

Our field data demonstrate that the majority of females become mated very rapidly after their maturation. These results match our second prediction, indicating higher competition for virgin females early in the season, and thus support the mating opportunity hypothesis for the evolution of protandry (Fagerström & Wiklund 1982; Morbey & Ydenberg 2001). Females seem however to mature throughout the season, so late-matured males could still access virgin females. Early-matured males are therefore likely to face higher levels of male-male competition than do males that mature later in the season. In closely related species of crab spiders with similar life-histories, late-matured females tend to remain unmated as the number of males drastically decreases towards the end of the season (Morse 2013a). In *M. celer*, however, the operational sex-ratio remains male-biased during the entire season. Such male-biased sex-ratio suggests that the intensity of male-male competition is constantly high, and does not decrease significantly towards the end of the season. If females mature asynchronously throughout the season, and access to virgin females is the only benefit in play, males may present a mixed strategy, with some males maturing early and securing copulations with virgin females at the beginning of the season, and some males maturing later and benefiting from the higher proportion of virgin females found in the last weeks of the season. Late-matured males, however, likely face other costs in addition to competition for virgin mates. First, mate-searching costs are likely higher in the end of the season, as the total number of females available in the population decreases drastically (Fig. 1B), increasing the difficulty of locating females on isolated flowers among dense prairie vegetation. Second, offspring from early-matured males are likely to hatch sooner and have a longer period of time to forage before entering winter diapause. Early-matured males may therefore benefit from greater offspring survival than late-maturd males, simply due to the timing of their offspring's birth. Finally, male life-span also influences the benefits obtained through protandry as it determines the length of the males' reproductive season (Wiklund & Fagerström 1977; Morbey & Ydenberg 2001). As sexual cannibalism is relatively infrequent in *M. celer*, males may mate multiply (Chelini & Hebets 2016) and, in laboratory conditions, can live for more than two months (MC Chelini *pers. obs.*). Early-maturation thus may grant males access to a larger number of virgin females throughout the season, and not simply in the days following maturation of the first females. Nonetheless, these results raise an interesting and seldom tested aspect of protandry - whether the benefits obtained by maturing early in the season could be offset by the intense competition for mates (Holzapfel & Bradshaw 2002; Kasumovic & Andrade 2009).

The variation in female and male *M. celer* size throughout the season is further evidence that protandry is not an indirect product of selection for SSD in this species (i.e. refuting the constraint hypothesis). *M. celer* spiderlings may be born from three weeks to three months after copulation occurs, depending on the female's food intake and on the number of egg sacs that a female lays (Chelini, *pers. obs.*). As such, in order to mature between early June and late July, spiderlings may have different amounts of time to develop depending on when were they born. In arthropod species with winter diapause, such as *M. celer*, late-born individuals are likely to be constrained to shorten and/or speed up their development to mature prior to the end of the reproductive season, and late-matured tend to be smaller than early-matured ones (Goulson 1993). Our results follow this pattern, with both males and females exhibiting smaller sizes at the end of the season. If both females and males regulate the timing of their development based upon the progression of season, they could also regulate their development in order to mature simultaneously. As such, it is unlikely that protandry evolved as a by-product of selection for SSD.

Interestingly, the variation of female and male growth trajectories also calls into question the mechanism(s) underlying the relationship between the mating advantage hypothesis and SSD: if females are able to accelerate their growth and reach 1.5 to 2 times the males' body size in approximately two weeks, males could likely reach larger sizes and still mature prior to females in the field (see theoretical predictions of Nylin *et al.* 1993). Small male size may therefore in itself be selected for through differences in survival (De Mas *et al.* 2009; Vollrath & Parker 1992), increased agility (Corcobado *et al.* 2010), or simply smaller foraging requirements (Blanckenhorn *et al.* 1995). Future research should explore these potential additional benefits of small male size. Ultimately, our maturation time and size data highlight the benefits of protandry in a species with female-biased SSD, but challenge the assumption that SSD in spiders is simply a consequence of selection for early male maturation (e.g. (Elgar & Bathgate 1996; Maklakov *et al.* 2004; Danielson-François *et al.* 2012).

Spiders are an ideal study systems to explore the relationships between female-biased SSD and protandry due to their frequently observed co-occurrence across diverse spider taxa, as well as the variety of mating systems and life histories in which these two traits are found. Our results with *M. celer* add further support to the notion that protandry is not a by-product of selection for large females size, but rather the main driver of female-biased SSD evolution in spiders (Kasumovic and Andrade 2009; Elgar & Bathgate 1996; Legrand & Morse 2000; Danielson-François *et al.* 2012; Neumann & Schneider 2015, but see Maklakov *et al.* 2004). Nonetheless, the hypothesized relationship between SSD and degree of protandry has been theoretically (Nylin *et al.* 1993) and empirically shown to be weak in a variety of arthropods species, including spiders (Cueva del Castillo & Nunez-Farfan 1999, our own results, see review by Blanckenhorn *et al.* 2007). We suggest that adaptive protandry is likely not the only factor leading to female-biased SSD, but small male size itself may provide fitness advantages. Integrative studies focusing on multiple sources of natural and sexual selection across a diverse set of taxa are required to clarify the relationship between protandry and female-biased SSD.

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