Received: 23 July 2021 | Revised: 26 January 2022 | Accepted: 8 February 2022

DOI: 10.1111/mec.16405

ORIGINAL ARTICLE

MOLECULAR ECOLOGY WILEY

Sperm competition intensity affects sperm precedence patterns in a polyandrous gift-giving spider

Magdalena Matzke[1] | Søren Toft[2] | Jesper Bechsgaard[2] | Astrid Pold Vilstrup[2,3] | Gabriele Uhl[4] | Sven Künzel[5] | Cristina Tuni[1] | Trine Bilde[2]

[1]Department of Biology, Ludwig Maximilian University of Munich, Planegg-Martinsried, Germany

[2]Department of Biology, Aarhus University, Aarhus C, Denmark

[3]Department of Molecular Biology and Genetics, Aarhus University, Aarhus C, Denmark

[4]General and Systematic Zoology, University of Greifswald, Zoological Institute and Museum, Greifswald, Germany

[5]Department for Evolutionary Genetics, Max Planck Institute for Evolutionary Biology, Plön, Germany

Correspondence
Cristina Tuni, Department of Biology, Ludwig Maximilian University of Munich, Planegg-Martinsried, Germany.
Email: cristina.tuni@bio.lmu.de

Funding information
Danish Council for Independent Research, Grant/Award Number: 4002-00328B; Erasmus+

Handling Editor: Lisette Waits

Abstract

Sperm competition drives traits that enhance fertilization success. The amount of sperm transferred relative to competitors is key for attaining paternity. Female reproductive morphology and male mating order may also influence fertilization, however the outcome for sperm precedence under intense sperm competition remains poorly understood. In the polyandrous spider *Pisaura mirabilis*, males offer nuptial gifts which prolong copulation and increase sperm transfer, factors proposed to alter sperm precedence patterns under strong sperm competition. First, we assessed the degree of female polyandry by genotyping wild broods. A conservative analysis identified up to four sires, with a mean of two sires per brood, consistent with an optimal mating female rate. Then we asked whether intense sperm competition shifts sperm precedence patterns from first male priority, as expected from female morphology, to last male advantage. We varied sexual selection intensity experimentally and determined competitive fertilization outcome by genotyping broods. In double matings, one male monopolised paternity regardless of mating order. A mating order effect with first male priority was revealed when females were mated to four males, however this effect disappeared when females were mated to six males, probably due to increased sperm mixing. The proportion of males that successfully sired offspring drastically decreased with the number of competitors. Longer copulations translated into higher paternity shares independently of mating order, reinforcing the advantage of traits that prolong copulation duration under intense competition, such as the nuptial gift. Sperm competition intensity enhances the impact of competitive sexual traits and imposes multiple effects on paternity.

KEYWORDS
copulation duration, fertilization outcome, mating order, microsatellite markers, paternity, sperm competition, sperm precedence

Cristina Tuni and Trine Bilde hared senior authorship

1 | INTRODUCTION

Polyandry, where females mate with more than one male within a reproductive cycle, causes fierce competition among males for fertilization of the eggs (Parker, 1970; Parker & Pizzari, 2010), and imposes selection on females to afford preferred males a fertilization advantage (Eberhard, 1996; Firman et al., 2017). The outcome of competitive interactions results in substantial variation among males in fertilization success, with major implications for trait evolution, coevolutionary processes, and population-level processes such as reproductive isolation and speciation (Pizzari & Wedell, 2013). Estimates of the number of sires and/or the number of mating partners contributing to stored sperm reveal wide prevalence of polyandry in wild populations, also in species that form permanent social bonds (Griffith et al., 2002; Taylor et al., 2014). While molecular markers have generated significant insights into the outcome of competitive interactions between males for fertilization (Taylor et al., 2014), the mechanisms that determine competitive fertilization outcomes remain complex and largely unresolved.

The selection pressure on males to maximize paternity success results in a wealth of adaptations that facilitate fertilization success (Simmons, 2019; Tourmente et al., 2011; Uhl et al., 2010; Waage, 1979; Wada et al., 2005). These include variation in sperm characteristics such as sperm motility (i.e., sperm velocity, the proportion of motile sperm in the ejaculate), sperm viability (i.e., proportion of live sperm in the ejaculate), and morphology (i.e., size, shape), to enhance sperm competitiveness and hence siring success (Fitzpatrick & Lüpold, 2014; Simmons & Fitzpatrick, 2012). A numerical advantage in sperm number is an important determinant of successful fertilizations (Birkhead & Pizzari, 2002; Parker, 1990; Parker et al., 1997; Parker & Pizzari, 2010), and favours investment in larger ejaculates/testes (delBarco-Trillo, 2011; Kelly & Jennions, 2011; Lüpold et al., 2020; Parker, 1982). Since copulation duration is often positively associated with sperm transfer, this is an important mechanism in facilitating paternity success (Engqvist & Sauer, 2003; Martin & Hosken, 2002; Pilastro et al., 2007; Rubolini et al., 2006). Mating order may also predict paternity success (Pischedda & Rice, 2012), as fertilization outcome may be biased towards the first or the last male to mate. Last male sperm precedence, where the last male to mate fertilizes most eggs (often referred to as P2, the proportion of offspring sired by the last male to mate in a double-mating trial), is common in insects and birds (Birkhead & Hunter, 1990; Simmons, 2019), while other systems show notable variation in fertilization patterns. For instance, spiders may exhibit first sperm precedence, last male sperm precedence or lack of priority patterns (Tuni et al., 2020). In mammals, where fertilization follows insemination as sperm is only viable for a short period in the female reproductive tract, there are no clear sperm priority patterns, with timing of ovulation and ejaculate size being the most relevant predictors of fertilization outcome (Ramm et al., 2005). In species that store sperm, patterns of sperm storage are important, that is, if sperm stratifies within the female reproductive tract, depending on female reproductive anatomy, it may confer advantage to the first sperm to enter or to the uppermost layer, giving raise to first and last male precedence respectively, in a first-in-first out or last-in-first-out fashion (Devigili et al., 2016). Female sperm storage organs are often simplified into "cul-de-sac" types (allowing a first in-last out scenario for sperm) as described for most insects, where the storage organ is usually placed at the dead end of an insemination duct (Simmons & Siva-Jothy, 1998), and "conduit" types suggesting a first-in-first-out scenario (Orr & Brennan, 2015).

Spiders are useful for investigating fertilization outcomes due to their polyandrous mating systems and reproductive morphology, which include paired genitalia and sperm storage organs (Eberhard, 2004). Spiders typically possess two types of female genital systems. Most entelegyne spiders have two bilaterally symmetrical copulatory openings and insemination ducts each leading to a distinct storage organ from which a fertilization duct leads to the fertilization site (oviduct) ("conduit" type). When the ducts are attached at opposite sides of the spermathecae first male sperm precedence is expected. Most haplogyne spiders have a single genital opening that functions as copulatory and oviposition opening, connected directly to the oviduct and from there to paired or multiple type storage organs ("cul-de-sac" type), suggesting last male sperm precedence (Austad, 1984; Foelix, 2010). Although first- or last-male priority patterns based on gross female reproductive morphology has been favoured for some time, empirical studies report large variation in second male fertilization success (P2) (Tuni et al., 2020), and female morphology is more diverse than described above (Uhl, 2000). Differences in copulation duration between mating partners appear to be an important factor determining relative paternity success, as reported in the orb-weaver *Argiope bruennichi* (Nessler et al., 2007; Schneider et al., 2006; Schneider & Lesmono, 2009), the wolf spider *Pardosa agrestis*, (Kiss et al., 2019) and the redback spider *Latrodectus hasselti* (Andrade, 1996). In the nephilid *Trichonephila edulis*, paternity success depends on duration and number of copulations, and is furthermore inversely related to male body size (Jones & Elgar, 2008; Schneider & Elgar, 2005; Schneider et al., 2000). These studies suggest that fertilization success and sperm precedence patterns are determined by a combination of relative sperm number (copulation duration) and male individual characteristics such as body size, in combination with female reproductive morphology.

In the spider *Pisaura mirabilis*, males increase mating success and prolong copulation time by offering a food donation, that is, a nuptial gift, to the female at mating. The gift consists of a silk-wrapped prey, which the female consumes while the male engages in copulation and sperm transfer (Bristowe & Locket, 1926). The presence of the nuptial gift and its characteristics such as larger size (higher nutritional value) (Albo et al., 2011; Bruun et al., 2003), or a thicker silk layer (Lang, 1996), facilitates longer copulations, which positively relates to sperm transfer (Albo et al., 2013). Laboratory studies show that females are polyandrous (Toft & Albo, 2015; Tuni et al., 2013; Tuni & Bilde, 2010), and may bias paternity towards gift-giving males through selective sperm storage (Albo et al., 2013). The nuptial gift may also function to overcome female resistance, which could result in suboptimal mating rates for females (Parker, 2006). A study investigating sperm priority patterns using sterile male technique

revealed first male priority in double-mated females supporting predictions based on entelegyne "conduit" type female reproductive morphology; however based on results with up to four matings in which the last male to mate gained a fertilization success comparable to P2 of double matings, the authors hypothesized that the last male may gain a constant share of paternity, which could shift sperm precedence from first to last male under strong sperm competition (Drengsgaard & Toft, 1999). This scenario would be consistent with the evolution and maintenance of the nuptial gift giving behaviour by paternity benefits.

In arthropods, sperm precedence patterns are analysed using sterile male techniques, where paternity is assessed in double mating experiments with a sterile (by radiation) and a nonsterilised male based on proportional embryo development (Bilde et al., 2009; Parker, 1970), or by use of molecular markers, such as microsatellite DNA markers for parentage assignment of multiply-sired clutches (Bretman & Tregenza, 2005; Griffith et al., 2002; Taylor et al., 2014; Tuni et al., 2012). Experimental studies investigating the factors affecting sperm precedence mainly employ double-mating designs, although mating with more than two males is common in many species. Relatively few of these studies have investigated fertilization outcomes when females mate with more than two partners (Lewis et al., 2005; Zeh & Zeh, 1994). Here, we investigated competitive fertilization outcomes in *P. mirabilis* by use of microsatellite markers. First, we estimated the degree of polyandry in three wild populations (Danish, German and Slovakian) by determining the number of sires in wild caught broods. This allowed us to assess the level of polyandry, and test whether natural mating rates in the field are consistent with optimal mating rates determined in controlled studies where females are not food limited (Toft & Albo, 2015). Second, we conducted an experimental study to assess the effect of male mating order on fertilization outcome (sperm priority patterns) by genotyping broods of females mated with two, four and six different males, to test whether first male priority (suggested by gross female morphology) breaks down when females mate with more than four males as proposed by Drengsgaard and Toft (1999). Furthermore, we determined the importance of copulation duration (sperm transfer) on paternity success, and the effect of male body mass on copulation duration and fertilization success, by controlling for nuptial gift size.

2 | MATERIALS AND METHODS

2.1 | Study 1: Estimating number of sires in the wild

2.1.1 | Sampling of broods

Pisaura mirabilis females lay eggs in an egg sac, which is first carried around and then placed in nursery webs attached to the vegetation. Females stay on the webs to guard egg sacs and hatched spiderlings for some days. We collected adult females with their hatched broods, from three locations, in Mols (Denmark, 12 broods), Munich (Germany, 13 broods) and Trnava (Slovakia, 16 broods), during June and July 2015. An additional seven broods without a guarding female were collected from Denmark in summer 2013. Brood sizes are shown in Table 2. One brood from Denmark was removed from the analysis due to parasitism of the brood.

2.1.2 | DNA extraction

All samples were transported to Aarhus University (Denmark) for molecular analyses and were frozen. DNA extractions of adult females (legs) and spiderlings (whole body) were conducted using the DNeasy Blood & Tissue kit (Qiagen) and the spin-column protocol. DNA was extracted from three legs from each female, while given the large brood sizes, the spiderlings of each brood were pooled together in groups of 10. Although pooled samples incur the risk of being less informative than individual samples due to the possibility of misinterpretation of rare alleles or noise, it allows genotyping a larger proportion of the brood relevant for arthropods with very large broods (Duran et al., 2015). Prior to extraction, tissues were covered with liquid nitrogen and crushed with a pestle in a 1.5 ml Eppendorf tube before adding 20 ml of Proteinase K and incubating for a minimum of three hours at a temperature of 56°C, steadily shaking. Subsequently, 4 µl RNAse A was added to obtain RNA-free genomic DNA, and samples were incubated at room temperature for 10 min, before performing the remaining steps according to the Animal Tissue Protocol. In an attempt to increase the final DNA concentration, elution was done with 50 µl of AE buffer instead of 200 µl. DNA concentration was measured with a Qubit 4 Fluorometer (Thermofisher).

2.1.3 | Microsatellite primers and genotyping

Samples were genotyped at 10 polymorphic microsatellite loci using fluorescent-labelled primers (Table 1) (DNA Technology, Denmark) that were specifically characterized for *P. mirabilis* using 454-pyrosequencing (described in Krehenwinkel et al., 2019) and in Methods S1). Summary statistics for all loci (number of alleles per locus, expected and observed proportion of heterozygotes, inbreeding coefficients), and tests for deviation from Hardy–Weinberg equilibrium are shown in Appendix S1. These summary statistics are relatively similar among populations. Polymerase chain reaction (PCR) amplification was done in multiplex; adult females in 10 plex containing all 10 primer pairs and spiderlings in 2 × 5 plex each containing five primer pairs (Table 1). Multiplex PCRs were designed with the use of MULTIPLEX MANAGER 1.2 software (Holleley & Geerts, 2009). The multiplex PCR amplification was performed in 30 µl reactions with Qiagen Type-it Microsatellite PCR kit. Both positive and negative controls were performed in each set of PCRs. PCR products were loaded on a 3% agarose gel, and samples with distinct bands were genotyped by Macrogen Inc., South Korea (dye

TABLE 1 Primer sequences, primer names, repeat (tretra, tretranuceotide; tri, trinucleotide), modifications and size ranges

Primer sequence (5′–3′)	Primer	Repeat	5′ Mod	Allele size
GGAATGCCAAAATTAACTGGTG ACTGTGTGTGCA TGTTGCTG	Psm14F Psm14R	Tetra	6-FAM	128–152
AACCTTAAGATTGATCACACGAA CAATCATCTTACTCAAGGGGTTC	Psm24F[a] Psm24R	Tri	PET	210–255
CATAGGGTAAGGGGCACACA AGCTAGCAGACGTTGGTTCG	Psm26F Psm26R	Tetra	VIC	308–372
GCCTTCTATGGAGACGGACA GCAGTGCTGTGAGCAAAGTC	Psm28F Psm28R	Tetra	VIC	196–268
TGTACACATTGACATCAAAAATACTTA TGGAACTTGCCGTCTATCAA	Psm41F[a] Psm41R	Tetra	NED	112–118
GGA TGGAAA TTGTTGAAGTCA T CGCCACA TGAGCTTGA TTC	Psm09F[a] Psm09R	Tri	VIC	135–162
TTTCACAGCTTGGGACAGTCT TAGCTGAGGTTCCGGAGAGA	Psm29F Psm29R	Tetra	PET	304–360
ATTTTGAGCCCATCAGCAAG TCTTTTTCGAAGAAATGCTTACA	Psm30F Psm30R	Tetra	6-FAM	292–364
TTGGGATATGGCCCAAATAA CTGAGGTTCCGGAGAGAGTG	Psm36F Psm36R	Tetra	6-FAM	200–260
A TGCTGAACTTTCGCAGTGA CTTAAAACCGCAAACCGAAA	Psm44F Psm44R	Tri	NED	213–264

Note: Primers are characterized for *Pisaura mirabilis*. All primers are used for estimating number of sires in the wild (study 1), with one multiplex mix including Psm14, Psm24, Psm26, Psm28 and Psm 41, and one Psm09, Psm29, Psm30, Psm36 and Psm44. For determining competitive fertilization outcomes (study 2) one multiplex mix included Psm14, Psm28, Psm30, Psm41 and one Psm09, Psm36, Psm44.

[a]Indicates primers from Krehenwinkel et al. (2019).

set; DS-33, filter set; G5, internal standard size marker; 500 LIZ). Genotypes were subsequently analysed using Genemapper 5, trial version (Applied Biosystems).

2.1.4 | Allele count

Loci that showed failed PCRs or absence of maternal alleles were removed from the analysis. Pooled samples of offspring constrain the use of parentage assignment software, for this reason we used manual allele count, a conservative method that analyses each locus separately. When estimating parentage using the allele count method, the maternal alleles were precluded, and the remaining alleles were regarded as paternal alleles. To obtain a conservative number of sires, the number of paternal alleles were then divided by two, since each mate can potentially pass on two different alleles. For broods from the Danish population collected without a female, the maternal genotype was deduced by interpreting alleles present in all samples as the maternal genotype.

2.1.5 | Statistical analyses

Detailed description of tests for genotypic disequilibrium, deviation from Hardy-Weinberg equilibrium, estimates of genetic diversity in the form of allele frequencies, observed heterozygosity (H_o) and expected heterozygosity (H_e), inbreeding coefficients F_{IS} and $R_{IS,}$ and F_{ST} and R_{ST} for population differentiation are reported in Appendix S1.

After testing residuals for normality using Shapiro–Wilk *W* test, we used a one-way ANOVA to test for differences in brood size between populations followed by a Tukey's post-hoc test and a Kruskal–Wallis test to analyse differences in the minimum mean number of sires between populations. Given that more pooled groups of spiderlings would be tested for large than for small broods, the chance of detecting various alleles in a brood may increase with brood size. We therefore used linear regression to test for correlation between the number of spiderlings per brood and the number of paternal alleles detected. Data was analysed using the software R version 4.0.3 (R Core Team, 2017).

2.2 | Study 2: Competitive fertilization outcome

2.2.1 | Animal collecting and maintenance

Subadult spiders were collected during the last two weeks of April 2018 from grass meadows surrounding the campus of the Ludwig-Maximilian University (LMU) in Martinsried (Germany), the same population as sampled in Study 1. Spiders were brought to the laboratory and placed individually in plastic vials (3 cm in diameter, 7.7 cm

in height), each provided with moss, and covered with sponge lids. All spiders were given an individual ID and were kept in the same laboratory at room temperature (approximately 23–25°C) and natural day-night photoperiod. Every 2–3 days they were sprayed with water, fed with a mixed insect diet (fruit flies, houseflies, and cricket nymphs) and checked for moults to assess sex and maturation. Spiders were assigned to mating treatment 10–14 days following their last moult to adulthood. During the course of the experiment, animals were fed every three days, but females were not fed the day before the matings to increase their propensity of gift acceptance and mating.

2.2.2 | Mating trials

Females were assigned to three treatments varying in the number of mating partners: they were mated twice (2-matings, $n = 22$), four times (4-matings, $n = 20$) and six times (6-matings, $n = 20$), each time with a different male. Females were mated once a day or twice with a 4–6 h gap in between matings. There was no significant difference in the interval of time in between matings between treatments (Kruskal Wallis test, $\chi^2 = 1.52$, df = 2, $p = .47$). To avoid sperm depletion or behavioural exhaustion males were mated at most once a day and fed after mating. Males were used multiple times (3.47 ± 0.09 times on average) with different females, and were equally distributed across treatments.

Prior to each mating trial, males were weighed using a Kern–Sohn digital scale (accurate to 0.001 g) to include male weight in the analyses. A female was placed into a transparent plastic tank (19.5 × 13.5 × 14.5 cm) with a paper towel on the bottom for a minimum of 30 min and was allowed to walk and leave silk draglines, which are known to elicit a sexual response in males (Beyer et al., 2018). Upon the removal of the female, a male was placed into the same tank and after 5–10 min was given a prey for nuptial gift construction, which consisted either of a small cricket nymph (*Acheta domesticus*) or a housefly (*Musca domestica*) previously measured for body length using a digital calliper to standardize prey size (6.56 mm ± 0.49). Once the male had caught the prey, he was given 30 min to initiate wrapping it in silk. Wrapping consists of a series of wrapping "bouts" and was considered finished if the male did not wrap for five consecutive minutes. If a male did not start wrapping within 30 min, the female was placed into the tank, as this induces male wrapping behaviour. If the male did not wrap or court within the following 10 min, he was returned to his housing vial and replaced with a different male.

Once a male had produced a silk-wrapped gift, the female was placed into to the tank and male-female interactions were observed. Males court females by displaying the gift in front of them, raising their first pair of legs, and vibrating their abdomen (Magris & Tuni, 2019). Once the female accepts the gift, the male enters the mating position by moving underneath her in an antiparallel position to reach the opening of the female reproductive organs with one of its sperm transfer organs, the pedipalps. Copulation duration was measured as the sum of all pedipalp insertion durations, as males could decouple their palps to switch and use the other palp, or females could interrupt the copulation. If the couple separated and the female kept hold of the gift, the mating was considered terminated; if the male retained the gift, he was given the opportunity to court again. If a male had not attempted to mate or even court the female within 45 min of placing them together, or if he did in fact attempt to mate but was rejected by the female, the trial was terminated, and the female tested with another male. In two cases, female aggressive behaviour during a mating trial led to the death of a male. Mated spiders were returned to their housing vials. All mating trials for an individual female were conducted within the same week.

2.2.3 | Egg-laying and hatching

After a female had mated either two, four, or six times, she was placed into a tank (19.5 × 13.5 × 14.5), where she was provided with moss, leaves and small sticks crossed for supporting the production of a nursery web. Every 1–2 days females were fed with a combination of laboratory raised prey (as above) and recently collected insects caught by sweeping in surrounding meadows to provide a diverse nutrient supply during the egg laying phase.

Females that produced an egg sac were no longer fed. During inspection, if a female dropped her egg sac, it was carefully given back to her with forceps. If she did not reaccept it or continued to drop it, she was fed again to solicit the production of a second egg sac. Tanks were kept under a string of heat producing light bulbs for some hours every day (Tuni et al., 2017). As soon as spiderlings hatched, they were carefully transferred into an empty tank by use of a soft brush and counted. Males, females, and spiderlings were placed in a 1.5 ml Eppendorf tube filled with 0.5 ml of ATL buffer (tissue lysis buffer, Qiagen) and stored at −20°C. If the number of hatched spiderlings was below 20, the female was fed, to allow her to lay a second egg sac. A total of 34 out of 62 females produced successful egg sacs (hatched spiderlings): 10 from the 2-matings group, 12 from the 4-matings group and 12 from the 6-matings group. A total of 25 females produced egg sacs with no hatching success and three females did not produce an egg sac (two from the 2-matings group, one from the 6-matings group). While most females produced a single successful egg sac ($n = 30$), two females from the 2-matings group and two females from the 6-matings group produced two successful egg sacs each, from which all spiderlings were included in the analyses (see details below).

2.2.4 | DNA extraction

All samples were transported to Aarhus University in Denmark in an electric cooling box (Dometic CoolFreeze CFX 50W) at a temperature of −20°C and stored at −80°C. DNA extractions of 106 adult spiders (34 females, 72 males) and of 571 spiderlings were conducted using the Qiagen DNeasy Blood & Tissue kit and the spin-column protocol, respectively from the cephalothorax and the whole body, as described above.

In order to reduce the number of extractions spiderlings were pooled. If the number of spiderlings per egg sac exceeded 60, 60 individuals were randomly chosen and pooled together in groups of three, leading to a total number of 20 extractions per brood. If the number of spiderlings per egg sac was below 60, 40 of those spiderlings were randomly chosen and pooled together in groups of two, leading to a total number of 20 extractions (or less) per brood. For adult spiders, 60 µl DNA yield were used (30 µl AE buffer during each elution), while for spiderlings a total of 35 µl DNA yield were used with 20 µl AE buffer in the first, and 15 µl in the second elution. DNA yields were frozen at −20°C until further use.

2.2.5 | Primers and genotyping

PCR amplification was done in multiplex; it was performed in 30 µl reactions with Qiagen Type-it Microsatellite PCR Kit. A negative control was performed in each set of PCRs. Adult spiders were genotyped at seven polymorphic microsatellite loci using fluorescent-labelled primers (Table 1), in 1 × 4 plex, containing four primer pairs (Psm14, Psm28, Psm30, Psm41) and 1 × 3 plex, containing three primer pairs (Psm09, Psm36, Psm44). For the spiderlings, only one plex was used, containing varying numbers of primers (between 1 and 5), depending on the alleles of the parents. If paternity could be assigned by assessing a single primer pair due to differing alleles in the mother and her mating partners, only this specific primer pair was used (2-matings group). In the 4- and 6-matings group a combination of 4–5 primers was used in all cases, Psm14, Psm41, Psm09, Psm36 and Psm44. PCR products were loaded on a 3% agarose gel and fragment analysis was done by Macrogen Inc. Since spiders were obtained from the same German population as Study 1, summary statistics for all loci are assumed to be the same as those presented in Appendix S1.

2.2.6 | Assigning paternity

Scoring of microsatellite markers was done with RStudio, 1.1.453, using the package "Fragman" (Covarrubias-Pazaran et al., 2016). Alleles of adult spiders were scored, and allelic peaks of each female were compared to the allelic peaks of the males she was mated with to identify alleles of their offspring and avoid scoring noise as potential real peaks. Noise is defined as stutter patterns or artefact peaks around real allelic peaks that do not inform on the individual's identity. Genotyping error rate, estimated by dividing the number of offspring samples in which the mother's alleles were both missing by the number of total offspring samples, was 5.56%. It was mainly driven by two of the five primers, namely Psm36 (6.18%) and Psm41 (16.9%), while error rates of primers Psm14 (3.16%), Psm09 (1.76%) and Psm44 (0.65%) were lower.

To assign the proportion of offspring sired by each male ("paternity success"), the following procedure was applied (see also Figure 1): The potential fathers were scanned for unique alleles (UA), which are peak(s) that do not reappear in the mother or in any of the other potential fathers, in each of the used markers. If such an allele was detected in the pooled samples of offspring, the male was declared the father of at least one of the spiderlings. If another male with a UA appeared in the sample, shared paternity was declared. Males which had matching alleles with the spiderlings in all the markers, however none of their allele matches were unique due to sharing these alleles with either the mother, or one of the other males, were labelled as males with "nonunique alleles" (NUA). If a pooled sample of offspring contained a UA and a NUA, these males were both declared fathers. Paternity was assigned to three different fathers in samples with three pooled spiderlings, if each male had either a UA, or a NUA. A male was declared the sole father, if no other UA or NUA was present in the sample. In the case of contradiction, unique alleles were scored with a higher priority than a non-unique match. If three spiderlings were pooled in one sample, but only two males were detected as potential fathers, paternity was shared equally, resulting in assigning 1.5 spiderlings to each father. As a result, each male was assigned an individual paternity score ("proportion of offspring"), calculated as the number of sired spiderlings divided by the number of spiderlings tested from the respective brood. In 82.1% of all cases, paternity could be assigned exclusively based on UA males, and in 16.5% of cases due to a combination of UA and NUA males. In the rare case (0.8%) that no UA male was present in the sample, paternity was assigned to NUA males only, or, in the even rarer case (0.6%) that neither UA nor NUA males were detectable, paternity was assigned to males with matching alleles with the spiderlings in all the markers except one (AEO in Figure 1). Paternity was assigned using UA in 100% of the samples in the 2-matings group, in 66.8% of the 4-matings group (and 30.5% using UA and NUA) and in 85.1% the 6-matings group (and 13.8% using UA and NUA).

In two cases within the 2-matings group paternity could not be assigned. In both cases the unique allele of one male appeared in all offspring, making him the certain father of at least 50% of the offspring. One allele of the second male was identical to the mother, thus showing up in all offspring as well, making him a potential, but not certain father of the other 50% offspring. Since the other microsatellite markers did not allow paternity assignment, another approach using probabilities was applied in these two cases (Appendix S2), where we estimated the likelihood of a 50:50 shared paternity being allocated assuming both fathers to be present in one sample of pooled spiderlings. In one other case within the 2-matings group, paternity could not be assigned, not even by using probabilities. This case was therefore excluded from further analysis.

2.2.7 | Statistical analysis

Analysis of variance was performed to assess variation in body mass between individuals in different treatments groups, and to test whether the number of hatched spiderlings differed among the three mating groups (2, 4, or 6 matings). Kruskal Wallis test was used to test for differences in interval duration between female matings between treatments.

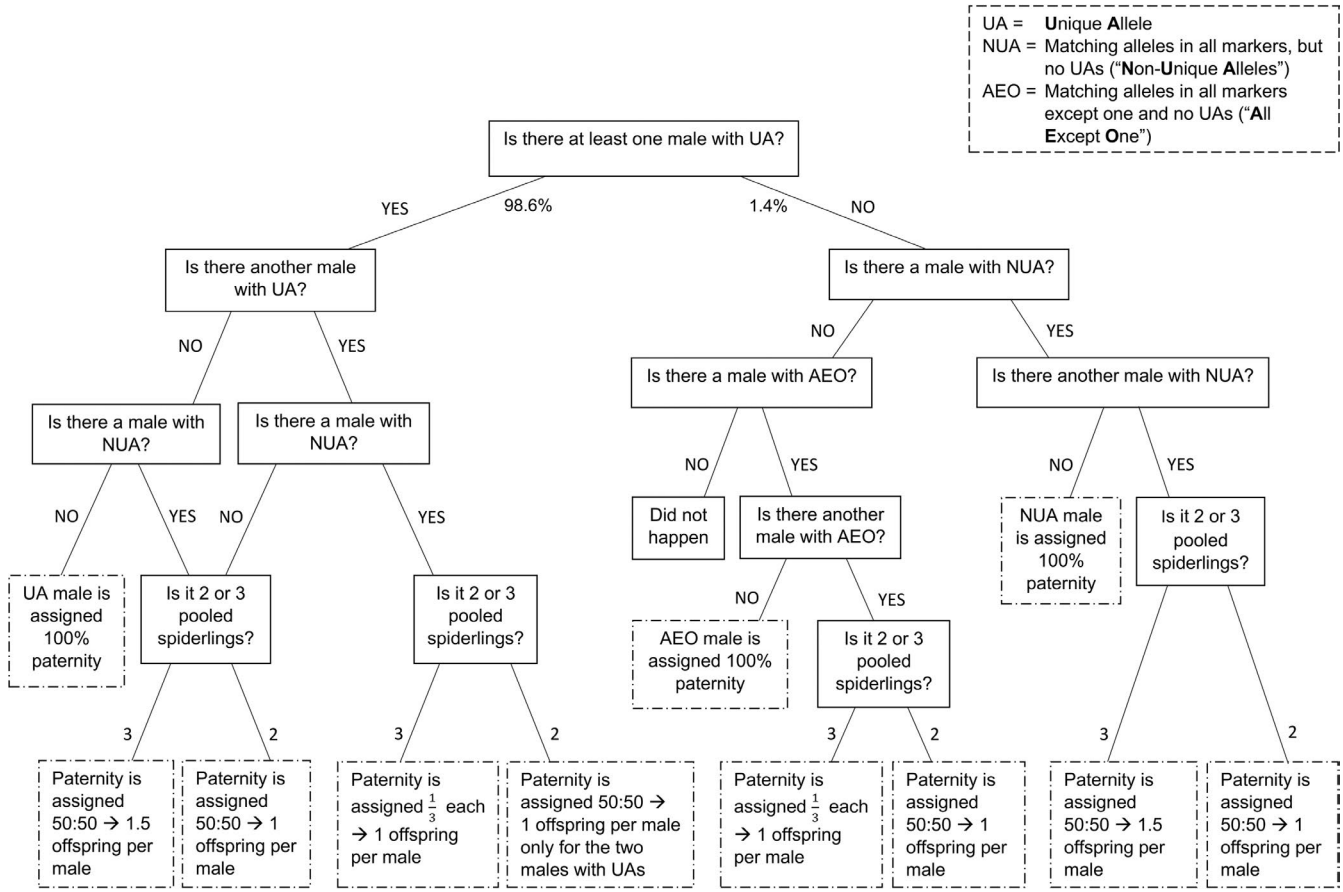

FIGURE 1 Flowchart depicting the procedure of paternity assignment through comparisons of scored alleles in all genetic markers between spiderlings, mothers, and potential fathers

A chi-squared goodness of fit test followed by a pairwise comparison (correcting for multiple comparisons) was used to determine whether the mean paternity success of each male (P1—PLast) significantly differed from an equal paternity share (25% in the four matings and 16.67% in the six matings), which is the predicted outcome of complete sperm mixing. To test whether successful versus unsuccessful males in the 2-matings group differed in bodyweight and copulation duration we used a nonparametric Wilcoxon signed rank test on paired samples.

To test whether copulation duration and mating order (mating positions of males 1–4 or 1–6) affect male paternity success (proportion of offspring sired by a male) we used generalised linear mixed effects models with a binomial distribution (glmer-b) for each mating group separately (4 and 6 matings). We excluded the 2-matings group from this analysis because paternity was never shared, one of the two males always sired all offspring with no priority to either first or second male to mate.

Male body mass was not included in this model, since we recovered a significant correlation between male mass and copulation duration. To test for first male advantage, we used the same model structure and contrasted paternity of the first male to mate with that of subsequent males to mate (pooled). Female ID was fitted as a random effect in all four models to account for pseudoreplication, and an individual observation-level ID (random effect) was included to correct for overdispersion. The same analysis was also conducted to assess potential last male advantage by contrasting paternity of the last male to mate to males mated previously (pooled), and is reported in Appendix S2. We additionally tested the effect of treatments (2-, 4- and 6-matings group) and copulation duration on paternity success (Appendix S2). Linear regression was used to determine the effect of male body mass on copulation duration and paternity success. Response variables were log transformed in case the distribution of residuals did not meet assumptions of normality. Data are reported in mean ± SE. All data was analysed in RStudio, using the package "lme4" (Bates et al., 2018) for generalised linear mixed models (logistic regression), the package "rstatix" (Kassambara, 2021) for pairwise comparisons, and the package "DHARMa" to assess model assumptions of the mixed effects models (Hartig & Hartig, 2017).

3 | RESULTS

3.1 | Study 1: Estimating number of sires in the wild

Multiple paternity was revealed in 17 out of 19 (89%) broods from Denmark, in 12 out of 13 (92%) broods from Germany, and in 11 out of 16 (69%) broods from Slovakia. In all three populations,

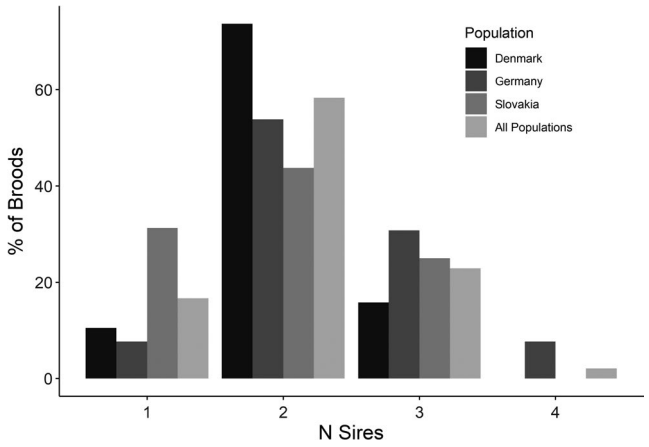

FIGURE 2 Proportion of the estimated number of sires for each *Pisaura mirabilis* population (Germany, Denmark, Slovakia) and for the three populations pooled (all populations)

the majority of broods were sired by two males (Figure 2), with the mean number of sires (±SE) being 2.05 (±0.12) in the Danish, 2.38 (±0.20) in the German, and 1.94 (±0.19) in the Slovakian population. No differences in the mean number of sires between populations were detected (Kruskal–Wallis test, $\chi^2 = 2.64$, df = 2, $p = .3$), instead we found differences in brood size between populations (mean number of spiderlings per brood; Denmark: 110.58 (±12.35), Germany: 183.85 (±25.08), Slovakia: 193.06 (±12.51), ANOVA, $F = 8.51$, df = 2, $p = .0007$; Tukey's post-hoc test, Denmark-Germany, $p = .008$, Denmark-Slovakia, $p = .001$, Germany-Slovakia, $p = .92$). While the number of loci genotyped for the Danish and German population ranged from 7 to 10, in four broods of the Slovakian population (broods 1–4) only up to four loci could be genotyped due to low quality samples, potentially affecting the ability to detect broods with multiple sires (Table 2). We found no correlation between brood size and the number of paternal alleles detected (Linear regression, $F(1,46) = 0.002$, $r^2 = 0.00005$, $p = .96$).

3.2 | Study 2: Competitive fertilization outcome

Males and females were randomly allocated to the three treatment groups and did not differ in their body mass across treatments (average male body mass 0.096 g (±0.004); 2-matings: 0.089 g (±0.008), $n = 18$; 4-matings: 0.096 g (±0.004), $n = 48$; 6-matings: 0.096 g (±0.003), $n = 72$; ANOVA: $F(2,135) = 0.72$, $p = .49$; average female body mass 0.096 g (±0.004); 2-matings: 0.087 g (±0.004), $n = 18$; 4-matings: 0.101g (±0.003), $n = 48$; 6-matings: 0.095 g (±0.003), $n = 72$; ANOVA: $F(2,135) = 2.43$, $p = .092$). The number of hatched spiderlings per female did not differ between treatments (average number of offspring 56.24 (±5.21); 2-matings, 61.9 (±9.57), $n = 9$; 4-matings, 60.33 (±9.37), $n = 12$; 6-matings 47.42 (± .68), $n = 12$; log-transformed; ANOVA, $F(2,31) = 0.86$, $p = .44$).

From the 72 males used in matings, 15 (20.8%) never produced offspring, regardless of their mating order, or the number of times they mated (2.9 ± 0.18). A total of 11 males out of the 15 were mated to females which failed to reproduce.

3.2.1 | Paternity outcome: 2-matings group

In the broods derived from trials with two mating partners, one of the two males always gained full paternity of the offspring (Figures 3a and 4a). The first males to mate (P1) sired the offspring in 56% (5 of 9 trials), while the second male to mate (P2) gained full paternity in 44% (4 of 9) cases. In eight of nine cases (89%) the siring male was also the one with longer copulation duration (Figures 3b and 5, Wilcoxon signed rank test on paired samples: $n = 9$, $V = 2$, $p = .018$). Successful and unsuccessful males did not differ significantly in body weight (Wilcoxon signed rank test on paired samples: $n = 9$, $V = 23$, $p = 1$, Figure 4b). There was a tendency for smaller males to achieve longer copulation duration, however this effect was marginally nonsignificant (Figure 4c). Males that did not sire offspring in the 2-matings group, however, sired offspring when reused in other treatments ($n = 6$).

3.2.2 | Paternity outcome: 4-matings group

Multiple paternity was detected in all broods of females mated to four mating partners (Figure 3c). 83.3% of the males successfully sired offspring, and paternity within broods was shared between an average of 3.33 (±0.14) males, ranging from 3 to 4. Specifically, the first male to mate had a relative paternity success of 0.348 (±0.068), second male to mate 0.156 (±0.033), third male to mate 0.329 (±0.077), and last male to mate 0.167 (±0.038). All four males significantly differed from an expected equal paternity share of 25%, as shown from the Goodness of Fit test ($\chi^2 = 64.3$, df = 3, $p < .0001$) as well as the pairwise comparison (Male 1: CI (95%) = 0.307–0.392, adjusted $p < .0001$; Male 2: CI (95%) = 0.125–0.190, adjusted $p < .0001$; Male 3: CI (95%) = 0.288–0.371, adjusted $p < .0001$; Male 4: CI (95%) = 0.136–0.203, adjusted $p = .0002$). First males to mate gained significantly higher paternity success compared with males in mating positions 2 and 4, but not compared to those in position 3 (Table 3, Figure 6a), and contrasting P1 to males 2–4 showed a significant first male advantage (Table 4). The analyses detected no significant advantage in paternity share for the last male to mate when contrasting males last in mating order with those mated previously (Appendix S2). Although not statistically significant, longer relative copulation duration tended to lead to higher paternity success (Tables 3 and 4, Figure 4d). In 50% of the cases, the male with the highest paternity outcome was also the one with the longest relative copulation duration (length of copulation duration relative to the other males mated to the same female, Figures 3c,d and 4d). Male body mass was a significant predictor of copulation duration ($R^2 = 0.062$, $F(1,46) = 4.09$, $p = .049$; Figure 4f), and regression analysis suggested a negative, however nonsignificant, effect of male body mass on paternity success ($R^2 = 0.015$, $F(1,46) = 1.74$, $p = .19$; Figure 4e).

TABLE 2 Estimation of the minimum number of sires in three *Pisaura mirabilis* populations

Population	Brood ID (number of offspring)	Number of loci genotyped	Number of paternal alleles	Minimum number of sires
Denmark	1 (44)	10	3	2
	2 (184)	10	4	2
	3 (134)	10	5	3
	4 (127)	9	6	3
	6 (120)	10	5	3
	7 (66)	9	3	2
	8 (70)	9	3	2
	9 (158)	10	2	1
	10 (126)	10	3	2
	11 (194)	9	3	2
	12 (26)	10	2	1
	13 (122)	10	3	2
	14 (164)[a]	9	3	2
	15 (64)[a]	10	3	2
	16 (15)[a]	6	4	2
	17 (136)[a]	10	3	2
	18 (83)[a]	10	3	2
	19 (181)[a]	10	3	2
	21 (87)[a]	9	4	2
Germany	1 (130)	9	3	2
	2 (140)	10	6	3
	3 (120)	10	4	2
	6 (265)	9	3	2
	7 (240)	10	3	2
	12 (358)	10	6	3
	13 (200)	7	2	1
	14 (297)	9	4	2
	16 (147)	10	3	2
	17 (16)	10	3	2
	18 (210)	10	7	4
	19 (154)	10	5	3
	20 (113)	10	5	3
Slovakia	1 (248)	2	1	1
	2 (230)	3	1	1
	3 (280)	4	1	1
	4 (200)	3	1	1
	6 (176)	9	3	2
	9 (146)	9	4	2
	10 (210)	10	5	3
	11 (180)	9	5	3
	12 (179)	10	6	3
	13 (240)	10	3	2
	14 (206)	10	5	3
	15 (87)	10	4	2
	16 (230)	7	2	1

(Continues)

TABLE 2 (Continued)

Population	Brood ID (number of offspring)	Number of loci genotyped	Number of paternal alleles	Minimum number of sires
	17 (179)	10	4	2
	19 (190)	10	3	2
	20 (108)	9	3	2

[a]Indicates broods collected without an adult female.

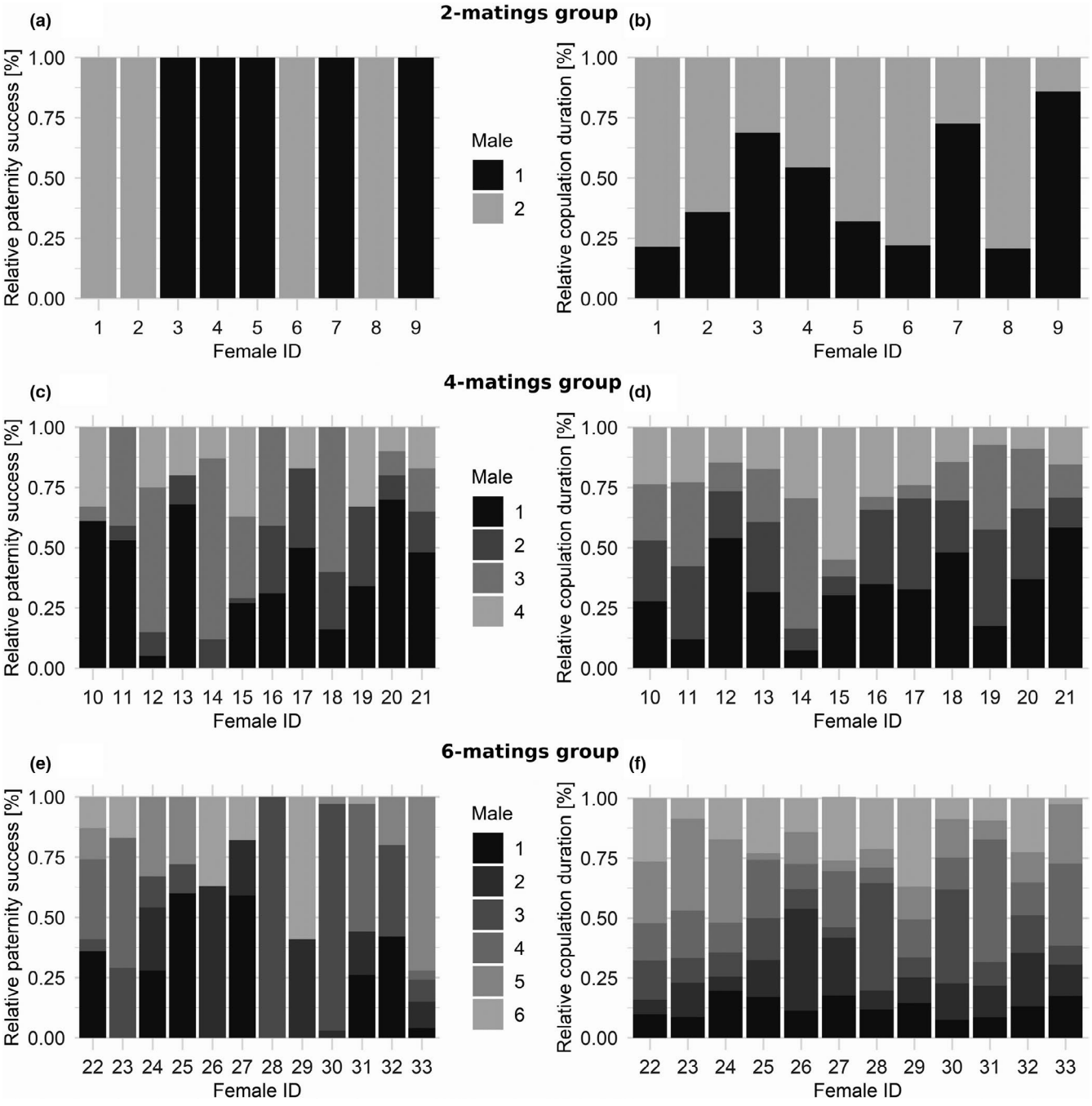

FIGURE 3 Relative paternity success (a, c, e) and relative copulation duration (b, d, f) of each male (1–6) per brood, in each of the mating groups (2, 4 and 6 matings)

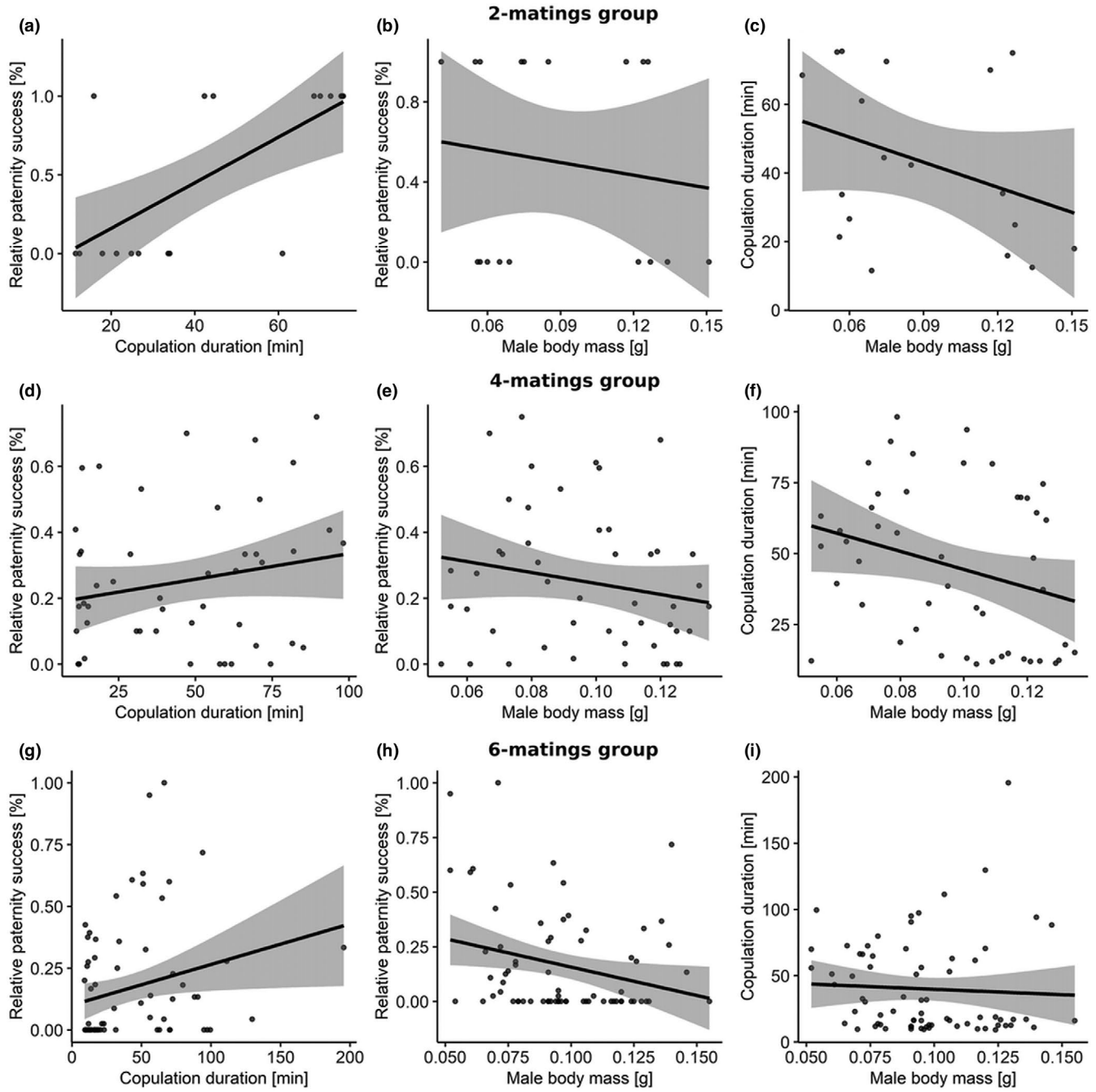

FIGURE 4 Effect of copulation duration (a, d, g) and male body mass (b, e, h) on relative paternity success and of male body mass on copulation duration (c, f, i) of the three mating groups (2, 4 and 6 matings)

3.2.3 | Paternity outcome: 6-matings group

Multiple paternity was detected in all broods of females mated to six mating partners (Figure 3e). In this group, only 52.8% of the males successfully sired offspring, suggesting that an increasing number of mating partners decreases the chance of siring any offspring. Paternity within broods was shared between an average of 3.17 (±0.34) males, ranging from 1 to 5. The first male to mate had a paternity success of 0.245 (±0.069), second male to mate 0.132 (±0.058), third male to mate 0.226 (±0.104), fourth male to mate 0.153 (±0.062), fifth male to mate 0.144 (±0.063), and last male to mate 0.101 (±0.055). All males significantly differed from an expected equal paternity share of 16.67% as shown from the Goodness of Fit test ($\chi^2 = 40.38$, df = 5, $p > .0001$). However, when conducting pairwise comparisons correcting for multiple comparisons males mated in positions 2, 4 and 5 did not significantly differ from the expected 16.67% (Male 2: CI [95%] = 0.101–0.168, adjusted $p = .176$; Male 4: CI (95%) = 0.120–0.191, adjusted $p = .475$; Male 5: CI (95%) = 0.112–0.181, adjusted $p = .433$) but males 1, 3 and 6 did (Male 1: CI [95%] = 0.205–0.288, adjusted $p = .0002$, Male 3: CI [95%] = 0.187–0.269, adjusted $p = .007$, Male 6: CI [95%] = 0.074–0.134, adjusted $p = .0008$). Mating order was no longer a significant

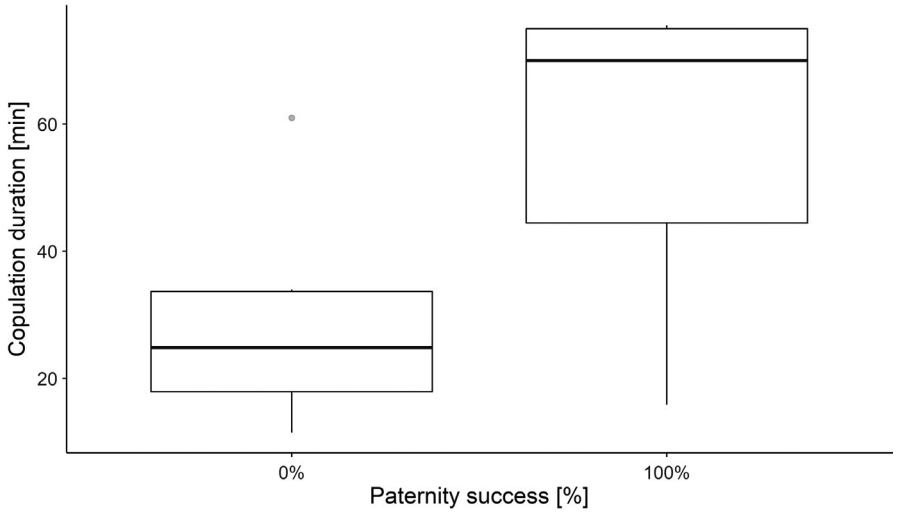

FIGURE 5 Copulation duration of unsuccessful (0%) and successful (100%) males in the 2-matings group

TABLE 3 Estimated effect sizes and 95% credible intervals around the mean of predictors of relative paternity success (glmer-b) in the 4- and 6-matings group; predictors: copulation duration, mating order effects indicated as contrasting mating positions nos. 2–4 or nos. 2–6 compared against mating position no. 1 (intercept)); random effects: Female ID, Observation-level ID. Copulation duration was standardized (centred). Significance shown in italics

	Four matings		Six matings	
	β estimate (95% CI)	***p*-value**	**β estimate (95% CI)**	***p*-value**
Fixed effects				
Intercept	−0.8 (−1.47, −0.157)	*.043*	−2.84 (−4.27, −1.38)	*.003*
Copulation duration	0.23 (−0.12, 0.56)	.276	1.12 (0.5, 1.73)	*.005*
Position no. 2	−1.2 (−2.11, −0.25)	*.041*	−0.35 (−2.38, 1.77)	.749
Position no. 3	−0.5 (−1.43, 0.47)	.396	0.78 (−1.32, 2.86)	.57
Position no. 4	−1.18 (−2.12, −0.2)	*.045*	−2.27 (−4.67, 0.07)	.097
Position no. 5			−1.24 (−3.27, 0.94)	.334
Position no. 6			−1.16 (−3.31, 0.99)	.368
	σ^2 (95% CI)		σ^2 (95% CI)	
Random effects				
Female ID	0.00 (0.0, 0.0)		0.00 (0.0, 0.0)	
Observation-level ID	1.98 (1.49, 2.56)		7.96 (6.09, 9.95)	
Residual	(π^2/3)		(π^2/3)	

predictor of paternity success (Table 3, Figure 6c), while longer copulation duration significantly predicted increased paternity success (Figure 4g, Tables 3 and 4). In 50% of the cases, the male with the highest paternity outcome was also the one with the longest relative copulation duration (Figures 3f and 4g). Contrasting paternity success of males in mating positions 2–6 with that of the first male to mate did not suggest significant first male advantage (Table 4), similarly, no significant advantage on paternity success was attributed to males last in mating order compared to those that mated previously (Appendix S2).

Linear regression showed a negative effect of male body mass on paternity success ($R^2 = 0.055$, $F(1,70) = 5.13$, $p = .027$, Figure 4h), however not on copulation duration ($R^2 = -0.01$, $F(1,70) = 0.21$, $p = .65$ (Figure 4i). Seven of the subsequently (2–6) mated males achieved above average paternity shares, of which five males additionally achieved the longest relative copulation duration compared to the other five mating partners.

When analysing the effects of the three mating groups and copulation duration on paternity share we found that paternity success decreases with the increasing number of competitors and increases with longer copulations (Appendix S2).

4 | DISCUSSION

4.1 | Polyandry in the wild

We detected multiple paternity in broods collected in three wild and geographically distinct *P. mirabilis* populations, confirming laboratory reports of females engaging in polyandry (Toft & Albo, 2015; Tuni et al., 2013; Tuni & Bilde, 2010). In the field, we identified up to four sires per brood, with the most frequent estimate in all populations being two sires per brood. If this number of sires also reflects the number of males the female actually mated with, it would be consistent with the optimal mating rate that was assessed under well-nourished conditions: females achieved the highest reproductive success (maximal egg hatching success) when mated with 2–3 males (Toft & Albo, 2015). Our findings on paternity share in study 2 may further support that

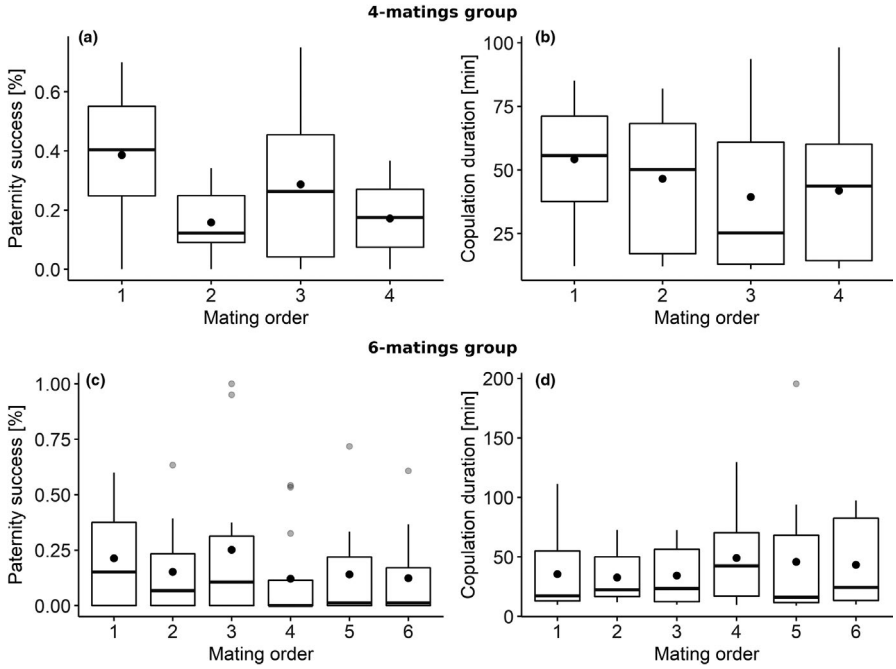

FIGURE 6 Relative paternity success (a) and copulation duration (b) of males mated in the 4-matings group according to their mating position (1–4); relative paternity success (c) and copulation duration (d) of males mated in the 6 matings group according to their mating position (1–6). Black circles represent arithmetic means

TABLE 4 Estimated effect sizes and 95% credible intervals around the mean of predictors of relative paternity success (glmer-b) in the 4- and 6-matings group; predictors: copulation duration, first to mate (intercept) contrasted against all subsequent males; random effects: Female ID, Observation-level ID. Copulation duration was standardized (centred)

	4 matings		6 matings	
	β Estimate (95% CI)	***p*-value**	**β estimate (95% CI)**	***p*-value**
Fixed effects				
Intercept	−0.8 (−1.49, −0.09)	0.052	−2.85 (−4.37, −1.71)	*.003*
Copulation duration	0.2 (−0.15, 0.56)	0.348	1.00 (0.394, 1.62)	*.011*
Subsequent	−0.97 (−1.79, −0.16)	*0.049*	−0.82 (−2.48, 0.83)	.426
	σ^2 (95% CI)		σ^2 (95% CI)	
Random effects				
Female ID	0.00 (0.0, 0.0)		0.00 (0.0, 0.0)	
Observation-level ID	2.11 (1.64, 2.65)		7.93 (6.21, 9.81)	
Residual	(π^2/3)		(π^2/3)	

the optimal number of sires is around three, as matings with four and six different partners resulted in an average of three males siring the broods. Polyandry may be driven by both direct and indirect benefits (Arnqvist & Nilsson, 2000; Slatyer et al., 2012), as well as by sexually antagonistic coevolution (Parker, 2006). In *P. mirabilis*, there is evidence for both direct material benefits from the nuptial gift and for indirect genetic benefits of polyandry that improve offspring viability (Toft & Albo, 2015; Tuni et al., 2013). However, Toft and Albo (2015) also revealed that food deprived females engage in up to 16 matings, indicating that food limitation changes the balance between mating costs and direct benefits via nourishment from gifts. The evolution of the nuptial gift, which is based on males exploiting the female foraging motivation to exchange nutrients for matings (Bilde et al., 2007), might have been favoured by fluctuations in food availability, as spiders often live under food limited conditions (Wise, 1983).

We identified up to four sires per brood, but these paternity estimates are conservative as genotyping may underestimate the number of males that sire offspring if the markers do not allow full resolution (shared alleles), and potentially from genotyping DNA extracted from pooled samples of offspring rather than individual offspring (Duran et al., 2015). In addition, not all males that mate actually sire offspring due to post-mating bias in sperm use (Albo et al., 2013; Bretman & Tregenza, 2005; Turnell & Shaw, 2015), or male infertility (García-González, 2004). Indeed, our experimental study showed that the proportion of males that successfully sired offspring decreased from 83% in the 4-matings group to 52% in the 6-matings group, suggesting that the number of mating partners in the field may be higher than the number of sires identified by genotyping wild broods. This may be particularly relevant for females mated with two males that, as shown from our study, always resulted in a single sire.

4.2 | Competitive fertilization outcome

4.2.1 | Sperm priority patterns

Fertilization success in relation to mating order was determined in staged mating trials varying in sperm competition intensity. If *P. mirabilis* females had a conduit type reproductive morphology, we would expect to find first male priority as in the previous study using sterile male technique (Drengsgaard & Toft, 1999). Data from trials with four males identified a mating order effect with first male precedence and a decreasing paternity share for males that mated subsequently. In trials with six matings, all males similarly gained a diminishing paternity share and the advantage to the first male weakened into nonsignificance. In both treatments, males in all mating positions could potentially acquire paternity. These results suggest a degree of sperm mixing (as inferred from shared paternity) overlaid by a mating order effect. Sperm from males in any mating position can enter the sperm storage organ, but the likelihood of each ejaculate of siring offspring decreased with increasing number of ejaculates. This might happen if an ejaculate is more likely to be pushed out of the genital system when females mate with more mating partners. Drengsgaard and Toft (1999) found higher first male precedence and higher P4 than the estimates obtained in our study, and they proposed a model of a relatively constant paternity share for the last male to mate, conferring increasingly stronger last male priority as the number of mating partners increase. The data obtained here does not support a reversal of the mating order effect. Instead, they indicate a gradual decrease in fertilization success with mating order, while confirming that males late in mating order may obtain a substantial fertilization success. Overall, this suggests that sperm competition intensity influences sperm precedence patterns.

We observed multiple paternity in the 4 and 6 male matings groups. Notably, as the number of mating partners increased, the paternity share for each successful male decreased, and a lower proportion of males were successful, demonstrating that the intensity of sperm competition strongly impacts the likelihood of fertilizing offspring. In fact, the number of sires were the same in the 4-matings and the 6-matings groups. Although only half of the males in the 6 male group succeeded in siring offspring, any male regardless of mating order could potentially gain a fair paternity share. Apart from male competitive advantages and/or female cryptic processes of sperm usage, the lack of fertilization success could be due to male infertility or insemination failure (García-González, 2004), possibly explaining the 20.8% entirely unsuccessful males in our experiment. However, the fact that we saw a reduction from 83 to 52% in male paternity success from the 4- to the 6-matings group suggests that at least some of the reduction in fertilization success results from increased rivalry. The combination of an elevated risk of gaining no paternity under high sperm competition with the fact that the last male to mate can achieve a fair paternity share (10%–16% in our study) suggests that sexual selection intensity interacts with adaptations that enhance fertilization success. Sperm competition is therefore likely to be essential for maintenance of the male gift giving trait that functions to prolong copulation duration.

The result from the 2-matings group differed from the other two treatments: in all trials, one of the two males obtained full paternity of the offspring, regardless of the order of insemination, and neither sperm priority patterns with first male advantage nor sperm mixing (shared paternity) was detected. Although we cannot rule out methodological issues for the observed bimodal distribution of P2 if the alleles of one of the two males remained undetected, it seems unlikely that this would occur in all samples. We can also exclude male sterility as an underlying explanation, as most of the males were also used in other mating trials, where they successfully sired offspring. Results in the 2-matings group could be caused by males succeeding in excluding sperm from the rival male, or alternatively, from females biasing paternity in favour of a preferred male (Albo et al., 2013). As we observed sperm mixing in trials with four and six matings, these mechanisms might break down when females mate with more than two males (see above). Change in sperm priority patterns based on the mating context are for example reported in pseudoscorpions, with the last male siring most offspring in double matings, whereas mixed paternity occurred in triple-matings (Zeh & Zeh, 1994). Alternatively, double-mated females may have been more likely to experience cryptic mating failure, for example, in the seed bug *Lygaeus simulans*, double-mated females were more likely to produce offspring sired by one of the two mating partners than expected by chance (Balfour et al., 2020).

Purely random effects relating to the degree of sperm mixing can also potentially lead to binomial patterns if each discrete ejaculate does not "break up" within the female reproductive tract (Harvey & Parker, 2000), that is, if sperm is stored in different compartments of the female storage organ. The structure of the female sexual organ (epigyne) in *Pisaura* may promote such random allocation of paternity as it may function as both conduit and cul-de-sac (Sierwald, 1989, and see Appendix S3). This morphology could also explain why an increasing number of males fail to fertilize any eggs with an increasing number of mating partners, if the sperm of some males gets overlaid by sperm from other males.

4.3 | Proximate factors influencing paternity success

Copulation duration was a strong predictor of fertilization success, which is perhaps not surprising as it is positively correlated with sperm transfer (Albo et al., 2013). For example, males that gained paternity in the 2-matings group experienced copulations approximately threefold longer than those that did not sire any offspring. In *P. mirabilis*, copulation duration longer than 10 min is decisive for successful sperm transfer, i.e. copulations shorter than 10 min result in insufficient sperm transfer to lead to successful fertilization (Albo et al., 2013). Mean

copulation duration for *P. mirabilis* is 42.4 (±2.7) min, which in addition to sperm transfer may function as copulatory courtship to facilitate fertilization (Eberhard, 1991; Edvardsson & Arnqvist, 2000). If copulation, which is largely under female control, is interrupted early, males will fail to accomplish sufficient sperm transfer to gain fertilization. This highlights the importance for males of prolonging copulation duration via the donation of nuptial gifts (Albo et al., 2011, 2013; Bruun et al., 2003), and may explain the evolution of wrapping the gift in silk as this prolongs female consumption time (Lang, 1996). Ghislandi et al. (2018) proposed that males may be under selection to provide genuine (as opposed to worthless) and larger prey gifts late in the mating season, when females are likely to have mated multiply, to improve success in sperm competition. However, this was not tested in our experiment where gift size was kept constant. While the alternative strategy of offering worthless gifts (empty exoskeletons) secures male mating success, the insertion time and thereby sperm transfer is shorter, as females terminate copulation faster when offered worthless gifts because they are consumed faster (Albo et al., 2011; Bruun et al., 2003). This supports worthless gift donation as a male mating strategy early in the season, possibly to secure first male advantage. In accordance, the proportion of worthless gifts held by males in the field was found to decrease over the mating season (Albo et al., 2019).

In certain groups (in particular the 4-matings group) we found a trend for copulation duration to be negatively correlated to male body mass, and for smaller males to gain relatively higher paternity shares. These results might be indicative of competitive advantages to small males. Despite not being the norm, a mating advantage for small males has been reported in many species across taxa for example, zebrafish (Watt et al., 2011), moorhens (Petrie, 1983), butterflies (Marshal, 1988), poecilid fishes (Bisazza & Pilastro, 1997) and Dipterans (McLachlan & Allen, 1987). The underlying explanation for the success of smaller males may rely on their higher agility and/or manoeuvrability and lower energy requirements (Blanckenhorn, 2000). Sexual selection favouring small male body size could explain the emergence of small males early in the season in a Danish *P. mirabilis* population (Albo et al., 2019), as the combination of small male size and protandry as a life history strategy (Maklakov et al., 2004) in concert would provide first male and small male advantages. Females are much more receptive to the first male than to subsequent males (Tuni & Bilde, 2010), which may facilitate longer copulations for the first male, potentially allowing the male to fill the female sperm storage organs to impede sperm transfer from future males.

In conclusion, our study demonstrates that polyandry is widespread in the wild as shown in a broad range of taxa (Taylor et al., 2014). If the number of sires identified in wild caught broods accurately reflects the number of males the female actually mated with, it would be consistent with an optimal mating rate for females. First male advantage and a mating order effect on fertilization outcome was present when females were mated to four males, but this effect vanished under more intense sperm competition. This leaves a substantial share of fertilizations for the last male, as expected for the nuptial gift-giving trait to be maintained. Copulation duration, and hence sperm transfer, was confirmed to be a generally strong predictor of fertilization success irrespective of mating order. Our study also suggests that sexual selection may act on body sizes in unexpected ways, as male size was negatively correlated to copulation duration suggesting small male advantage. As the number of mating partners increased, the paternity share for each successful male decreased, and a lower proportion of males were successful in gaining paternity. This demonstrates that the intensity of sperm competition impacts on sperm precedence patterns by excluding some sires and increasing the relative success of males that are in the last mating positions. The competitive ability of an ejaculate is enhanced by increasing sperm numbers via prolonged copulations, and this effect seems to be exacerbated under intense sperm competition.

ACKNOWLEDGEMENTS

We thank Paolo Giovanni Ghislandi for collecting spiders for study 1, Daniel Heimerl for his valuable help in collecting and rearing animals for study 2, and Annie Brandstrup for assistance with the molecular work. The sequencing was performed in the MPI for Evolutionary Genetics in Plön, Germany. Thanks are due to Susanne Meese and Silke Fregin (both at Greifswald University) for developing the microsatellite markers. This work was funded by The Danish Council for Independent Research grant number 4002-00328B to Trine Bilde and an Erasmus+ scholarship to Magdalena Matzke. Open access funding enabled and organized by ProjektDEAL.

AUTHOR CONTRIBUTIONS

Trine Bilde, Jesper Bechsgaard and Astrid Pold Vilstrup designed study 1, Trine Bilde, Søren Toft, Cristina Tuni and Magdalena Matzke designed study 2, Magdalena Matzke and Astrid Pold Vilstrup performed data collection, Sven Künzel, Gabriele Uhl and Jesper Bechsgaard supervised the development of markers, Magdalena Matzke and Astrid Pold Vilstrup analysed the data, Cristina Tuni, Magdalena Matzke, Trine Bilde wrote the initial manuscript draft, all authors contributed to developing the final manuscript.

DATA AVAILABILITY STATEMENT

Genotype data have been made available on DataDryad https://doi.org/10.5061/dryad.kwh70rz5f.

ORCID

Cristina Tuni https://orcid.org/0000-0002-7190-1143

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How to cite this article: Matzke, M., Toft, S., Bechsgaard, J., Pold Vilstrup, A., Uhl, G., Künzel, S., Tuni, C., & Bilde, T. (2022). Sperm competition intensity affects sperm precedence patterns in a polyandrous gift-giving spider. *Molecular Ecology*, 31, 2435–2452. https://doi.org/10.1111/mec.16405