

**Mating strategies and inbreeding in the  
orb-weaving spider genus *Argiope* (Araneae: Araneidae)**

**Dissertation**

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**Use of English in thesis by Stefanie M. Zimmer**

To whom it may concern,

As a native English speaker I am writing in support of Stefanie M. Zimmer's thesis entitled  
**"Mating strategies and inbreeding in the orb-weaving spider genus *Argiope*".**

Having read through Stefanie's thesis I can confirm that the use of English is correct.

Yours sincerely,

A handwritten signature in black ink, appearing to read 'Nadia Ezzat', with a stylized, cursive script.

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## Zusammenfassung

Paarungssysteme werden über die Paarungsraten von Weibchen und Männchen definiert. Monogyne Paarungssysteme zeichnen sich dadurch aus, dass sich Männchen trotz fehlendem elterlichen Investment nur mit einem (Monogynie) oder maximal zwei Weibchen (Bigynie) verpaaren, während die Weibchen hingegen mehrere Paarungspartner haben. Monogynie widerspricht daher der allgemeinen Auffassung, dass sich Männchen mehrfach verpaaren, während Weibchen wählerisch sind und sich nur mit dem besten Männchen paaren. Monogynie hat sich mehrmals unabhängig bei Spinnen entwickelt und geht mit einem maximalen und oft zeitlich begrenzten Paarungsaufwand der Männchen einher, sowie mit speziellen Anpassungen, wie z.B. Selbstaufopferung, Genitalverstümmelung und lebenslanger Bewachung des Paarungspartners.

Dieser hohe Paarungsaufwand weist darauf hin, dass Männchen gegenüber der Wahl ihres Paarungspartners nicht gleichgültig sein sollten. Weibchenqualität ist durch ihren Paarungsstatus, ihre Körpergröße und der damit verbundenen Fruchtbarkeit, sowie durch ihr Alter und durch die Kompatibilität zwischen den Paarungspartnern bestimmt. Ich habe monogame Spinnen der Gattung *Argiope* untersucht und Feld- und Laborexperimente mithilfe von verhaltensbiologischen und genetischen Methoden kombiniert. In den ersten vier Kapiteln konzentriere ich mich insbesondere auf die Ursache von genetischer Inkompatibilität, d.h. dem Vorkommen von Inzucht durch Verpaarungen zwischen Geschwistern und auf die Anpassungen zur Inzuchtvermeidung. In den letzten zwei Kapiteln konzentriere ich mich auf die männliche Präferenz der anderen oben genannten Merkmale, in dem ich binäre Wahlmodelle verwendet habe.

In den ersten zwei Kapiteln bestimme ich die genetische Zusammensetzung von Feldpopulationen von *Argiope bruennichi* in Raum und Zeit, um das Inzuchtrisiko in der Natur und die Konsequenz von Inzucht einschätzen zu können. Daten von verschiedenen Populationen haben eine überraschend hohe genetische Variation und ein moderates Inzuchtrisiko

aufgrund fehlender Populationsstrukturen gezeigt. Allerdings führte eine Verpaarung zwischen Geschwistern schon nach einer Inzuchtgeneration zu einem reduzierten Paarungserfolg. Somit scheinen die Kosten von Inzucht insgesamt groß genug zu sein, so dass Inzuchtvermeidungs-Prozesse selektiert werden.

Die folgenden Laborexperimente, die ich zur Bestimmung von Inzuchtvermeidungs-Mechanismen verwendet habe, basieren auf dem komplexen Aufbau des Geschlechtsapparates von *Argiope*, der bestimmte Limitierungen der Paarungsraten mit sich bringt. Daher ist es wichtig, die Details der Sexualbiologie von *Argiope* zu verstehen.

Männchen verstümmeln während der Paarung ihre paarigen Geschlechtsorgane (Pedipalpen) und benutzen das abgebrochene Geschlechtsteil, um die Geschlechtsöffnung des Weibchens zu blockieren. Dies begrenzt Männchen auf zwei Paarungen in ihrem Leben. Daher können sich Männchen entweder zweimal mit demselben Weibchen oder je einmal mit zwei verschiedenen Weibchen verpaaren. Der männliche Geschlechtsapparat und die weiblichen Genitalöffnungen erlauben nur ein festgelegtes ipsilaterales Befruchtungsmuster. Das bedeutet Männchen können nur mit ihrem rechten Pedipalp in die rechte Geschlechtsöffnung und mit ihrem linken Pedipalp in die linke Geschlechtsöffnung des Weibchens inserieren.

Im Allgemeinen reicht eine Paarung aus, um alle Eier des Weibchens zu befruchten. Da Mehrfachpaarungen gewöhnlich mit Kosten für das Weibchen verbunden sind, sollten sich Weibchen nur mit einem nicht-verwandten Männchen erneut verpaaren, um somit die Kosten durch genetische Vorteile auszugleichen. Damit einhergehend ist bekannt, dass Weibchen kryptisch ein nicht-verwandtes Männchen gegenüber einem Bruder bevorzugen, indem sie die Anzahl der gespeicherten Spermien der Paarungspartner kontrollieren. Weibchen besitzen zwei getrennte Spermien-speicher (Spermatheken), die jeweils unabhängig mit Spermien von unterschiedlichen Männchen während zwei verschiedenen Paarungen gefüllt werden können. Weibchen

sind sehr aggressiv und kannibalisieren die meisten Männchen bereits nach der ersten Paarung. Dadurch verhindern sie, dass sie von den Männchen monopolisiert werden. Dies ermöglicht eine weitere Befruchtung mit einem zweiten Männchen in die übriggebliebene Spermathek und erlaubt den Weibchen die Vaterschaft nach der Kopulation zu beeinflussen.

Männchen hingegen erhöhen ihren Reproduktionserfolg, indem sie ein Weibchen monopolisieren. Dies führt zu einem sexuellen Konflikt über die Paarungsraten. Da aber Inzucht in diesem Paarungssystem auch mit Kosten für das Männchen verbunden ist, entscheiden sie sich für eine bigyne Paarungsstrategie, wenn sie ihre erste Kopulation mit einer Schwester hatten. Wenn sie sich mit einer Schwester verpaaren, kopulieren sie kürzer und erhöhen dadurch die Chance dem Kannibalismus zu entkommen und können somit nach einem genetisch passenden Weibchen suchen.

Kryptische Weibchenwahl und fakultative Bigynie als Mechanismus zur Inzuchtvermeidung sind adaptiv, da Inzuchtdepression anscheinend einen negativen Effekt auf diese Spinnen hat. Die Vermeidung von Inzucht könnte ein wichtiger Selektionsdruck sein, der die Paarungsstrategien beider Geschlechter in dieser Gattung entscheidend prägt.

Im 3. Kapitel teste ich, ob *Argiope bruennichi* Polyandrie strategisch als Mechanismus zur Inzuchtvermeidung verwendet. Weibchen locken Männchen durch Pheromone an, jedoch werden sie für gewöhnlich nach der ersten Verpaarung unattraktiv. Um zu verstehen, ob Weibchen die Produktion von Pheromonen kontrollieren können um weitere Paarungen zu vermeiden oder zu erlangen, habe ich ein Experiment durchgeführt, in dem die Männchen anhand von Spinnenseide zwischen zwei Weibchen wählen konnten.

Nachdem Weibchen sich mit einem Bruder verpaart haben, wäre es für sie von Vorteil weiterhin Attraktivität auszustrahlen, um eine zusätzliche Paarung mit einem Nicht-Geschwister zu erhalten. Ich habe herausgefunden, dass Weibchen, die ihre erste Paarung mit einem Bruder hatten, attraktiver für ein zweites Männchen waren als die Weibchen, die bereits eine Paarung mit einem Nicht-Geschwister hatten. Diese Ergebnisse unterstützen die Idee, dass Weibchen

strategisch ihre Paarungsstrategien anpassen und Sex-Pheromone produzieren, wenn zusätzliche Paarungen von Vorteil sind.

Mechanismen von Inzuchtvermeidung vor der Kopulation scheinen bei Weibchen nicht vorhanden zu sein, da sie wahllos das erste Männchen akzeptieren; höchstwahrscheinlich um einen kompletten Reproduktions-Misserfolg zu vermeiden. Die „trade-up“ Hypothese prognostiziert, dass zusätzliche Paarungen mit einem Männchen vollzogen werden sollten, wenn dieser im Vergleich zum ersten Paarungspartner genetisch besser ist. Im 4. Kapitel teste ich diese „trade-up“ Hypothese als einen Mechanismus zur Vermeidung von Inzucht in *Argiope lobata*. Ich habe verpaarte Weibchen selektiv mit zwei Männchen unterschiedlicher Verwandtschaft (Bruder oder Nicht-Geschwister) in mehreren Kombinationen verpaart. Beide Geschlechter sollten mehr gewillt sein den zweiten Paarungspartner zu akzeptieren, wenn dieser verglichen zu dem ersten Paarungspartner genetisch besser ist. Wie prognostiziert habe ich herausgefunden, dass beide Geschlechter den ersten Paarungspartner widerstandslos akzeptierten. Gemäß der „trade-up“ Hypothese waren beide Geschlechter abgeneigt, sich mit einem Geschwister in den nachfolgenden Paarungen zu paaren. Allerdings, im Widerspruch zu der Hypothese hatte die Qualität des ersten Paarungspartners keinen Einfluss darauf, ob weitere Paarungen akzeptiert wurden. Das deutet daraufhin, dass Individuen dieser Art generell Geschwister als Paarungspartner vermeiden, nachdem sie bereits Spermien für die Befruchtung ihrer Eier gesichert haben.

Im 5. Kapitel zeige ich, dass *A. bruennichi* Männchen ihre Paarungsstrategien an die Verfügbarkeit und sich verändernde Qualität der Weibchen im Laufe der Paarungssaison anpassten. Die Anwesenheit eines Konkurrenten hatte dabei keinen Einfluss auf ihre allgemeinen Paarungspräferenzen. In der Regel bevorzugten Männchen jungfräuliche Weibchen, da die ersten Männchen aufgrund von Begattungspfropfen (engl. „mating plugs“) eine Spermienpriorität hatten. Waren allerdings Männchen älter und die Paarungssaison fortgeschritten, bevorzugten sie eher fruchtbare (d.h. schwere) Weibchen, selbst wenn diese bereits schon verpaart waren. Schwere Weibchen befinden sich kurz vor der Eiablage und überleben höchstwahrscheinlich



bis zur Eiablage. Somit sichern sich Männchen immer noch 50% der Vaterschaft, wenn sie sich mit einem bereits verpaarten, aber fruchtbaren Weibchen paaren.

Im 6. Kapitel untersuche ich die Paarungspräferenzen von Männchen in der Wespen spinne *Argiope keyserlingi*, abhängig von der Spermienkonkurrenz und dem Vorkommen von Begattungspfropfen. Ich habe gezeigt, dass Männchen zwischen einfach verpaarten und doppelt verpaarten Weibchen unterscheiden konnten und jeweils das Weibchen bevorzugten, welches sich nur ein Mal verpaart hatte. Männchen müssten einen Begattungspfropfen entfernen, um sich mit einem doppelt verpaarten Weibchen zu paaren. Verglichen mit einer Paarung mit einem einfach verpaarten Weibchen, müssten Sie im Erfolgsfall allerdings immer noch einen geringeren Vaterschaftsanteil erwarten. Wenn jedoch einfach ver-

paarte Männchen ein Paar von einfach verpaarten Weibchen angeboten wurde, deren Genitalöffnung entweder links oder rechts verstopft waren, konnten sie das für sie passende Weibchen nicht erkennen.

Abschließend lässt sich sagen, dass Paarungsstrategien in monogamen Paarungssystemen der Gattung *Argiope* entscheidend von Mechanismen zur Inzuchtvermeidung geprägt werden. Das in der Natur vorkommende moderate Inzuchtrisiko und die damit einhergehenden Kosten scheinen dabei treibende Kräfte zu sein, die die Paarungsstrategien entscheidend prägen. Des Weiteren sind die Variabilität der Weibchen-Qualität und Weibchen-Verfügbarkeit höchstwahrscheinlich eine treibende Kraft in der Entwicklung von männlichen Paarungspräferenzen in der Gattung *Argiope*.



## Summary

Mating systems are defined through female and male mating rates. Monogynous mating systems are characterised by males that provide no parental investment but nevertheless mate with only one (monogyny) or maximally two females (bigyny) and multiply mating (polyandrous) females. Monogyny contradicts the general perception that males should mate multiply, while females are selective and mate with the best male only. Monogyny has evolved several times independently in spiders and involves a maximal and often terminal mating effort of males along with curious adaptations such as self-sacrifice, genital mutilation and life-long mate guarding.

This high mating investment suggests that males should not be indifferent to the quality of their mating partner. Female quality is determined by her mating status, body size and related fecundity, age, and by the compatibility between mating partners. I studied monogynous spiders of the genus *Argiope* and combined field and laboratory studies using behavioural and genetic methods. In the first four chapters, I focused on a particular cause of genetic incompatibility, namely the occurrence of inbreeding through copulations between siblings and adaptations to avoid inbreeding. In the last two chapters, I focused on male preferences of the other traits using binary choice paradigms.

In **chapter 1 and 2**, I determine the genetic composition of field populations in space and time to evaluate the natural risk of incest and assess the consequences of inbreeding. Data from several populations revealed that genetic variation was surprisingly high and the risk of inbreeding modest due to a lack of population substructure. However, a single mating among siblings resulted in reduced hatching success already after one generation of inbreeding. Thus, these high costs of inbreeding might overall be large enough to select for inbreeding avoidance mechanisms.

To understand the following laboratory experiments, which I used to assess inbreeding avoidance mechanisms, it is important to appreciate the details of *Argiope* sexual biology because certain limitations create a complex

setting to which male and female optimal mating rates should be adjusted.

Males damage their paired genitals (pedipalps) during mating and use the broken genital part to plug up female genital openings. This restricts males to at most two copulations in their lifetime. Therefore, males can mate either with the same female twice or with two different females. The male genital apparatus as well as the female genital openings only allow a fixed ipsilateral insemination pattern, i.e. males can only insert their right pedipalp into the females' right genital opening and the left pedipalp into the left genital opening.

Usually, a single act of mating is sufficient for fertilisation of all female eggs. Since multiple mating is commonly associated with costs for the female, they should only re-mate with an unrelated male to outweigh these costs by indirect genetic benefits. In this case, females are known to cryptically favour unrelated males over siblings by controlling the number of stored sperm from mating partners. Females possess two separate sperm storage organs (spermathecae) that each can be filled independently with sperm from a different male during two different copulations. Females are very aggressive and cannibalise most males already after the first mating. Thereby females prevent males from monopolisation and facilitate another insemination by a second male in the remaining spermatheca, which allows females to post-copulatorily bias paternity.

Males on the other hand maximise their reproductive success by monopolising a single female, which suggests sexual conflict over mating rates. However, inbreeding is also costly for males in this system and therefore males opt for a bigynous strategy if they have had their first copulation with a sibling female. When mating with a sibling, males copulate shorter, which enhances their chance to survive a cannibalistic attack from the female and to search for a more compatible mate.

Cryptic female choice and facultative bigyny as inbreeding avoidance mechanisms are highly adaptive because inbreeding depression seems to affect these spiders. Hence, inbreeding

avoidance might be a selective force shaping the mating strategies of both sexes in this genus.

In **chapter 3**, I test whether *Argiope bruennichi* strategically use polyandry as an inbreeding avoidance mechanism. Females attract males with sex pheromones but usually become less attractive after their first copulation. To understand whether or not females can control pheromone production in order to avoid or facilitate further matings, I used a silk-based choice experiment.

After mating with a sibling, females may benefit from re-advertising their receptivity to secure outbreeding. I found that females that had received their first copulation from a sibling were more attractive to a second male than those females that had already mated with an unrelated male. These results support the idea that females strategically adapt their mating strategy and produce sex pheromones when additional matings seem beneficial.

Pre-copulatory inbreeding avoidance mechanisms seem to be absent in females and they accept their first mates indiscriminately probably to avoid a complete reproductive failure. The trade-up hypothesis predicts that additional matings should only be received from mates that are genetically superior to the first. In **chapter 4**, I test the trade-up hypothesis as an inbreeding avoidance mechanism in *Argiope lobata*. I selectively mated females with two males of different relatedness (sibling or non-sibling) in varying combinations. Both sexes should be more willing to accept a second mate if the latter is genetically superior compared to the first mate. As predicted, I found that both sexes accepted their first mate without discrimination. In accordance with the trade-up hypothesis, they were extremely reluctant to mate with a sibling in successive matings. However, contradictorily to this hypothesis, the quality of the first mate had no influence on the acceptance of further matings. This

indicates that after securing sperm for fertilisation, individuals of this species generally reject siblings as mating partners.

In **chapter 5**, I show that *A. bruennichi* males adapted their mating strategies according to the availability and varying quality of females in the course of the mating season, while the presence of competitors did not affect the general mating preferences. Generally, males preferred virgin females because first males have a sperm priority due to mating plugs. However, older males like in an advanced mating season preferred fecund (= heavy) females, even though these were already mated. Heavy females are close to oviposition and likely survive until egg-laying. Thus, males may still secure 50% paternity when mating with an already mated, but fecund female.

In **chapter 6**, I study male mate preferences in *Argiope keyserlingi* depending on sperm competition and the presence of mating plugs. I found that males could distinguish between single- and double-mated females and preferred those that had only copulated once. Males would need to remove a mating plug to copulate with a double-mated female but would still expect a lower paternity share compared to mating with a single-mated female. However, if single-mated males were offered a pair of single-mated females whose genital opening was either plugged left or right, respectively, males were unable to detect the matching female.

In conclusion, I show that the mating strategies in monogynous mating systems of *Argiope* are likely shaped by inbreeding avoidance mechanisms. The naturally occurring modest inbreeding risk and the costs associated with it might be the driving forces shaping these mating strategies. Furthermore, the variability of female quality and availability, as well as sperm competition likely drive the evolution of male mate preferences in *Argiope*.

## General introduction

### Mating strategies and inbreeding

Mating systems occur in a wide variety in the animal kingdom and their evolution depends on the interaction of male and female mating strategies, which are each shaped by sexual selection (Shuster and Wade 2003). The relative strength of sexual selection can be different for the sexes resulting in different male and female mating strategies. Darwin was the first to recognise that males generally compete for access to females and mate indiscriminately with any available mating partner, while females do not simply mate with the first male they encounter, but rather seem to be choosy (Darwin 1871). The recognition of these classical sex roles with competitive, indiscriminate males and choosy females has been explained on the basis that females and males differ in their gamete size and hence in their investment into reproduction (Parker et al. 1972). Males produce numerous small, cheap spermatozoa and are able to fertilise eggs of several females; thus maximising their reproductive success by mating with as many females as possible. Females on the other hand produce only few large and costly eggs and do not increase their reproductive success by mating with more than one male. These fundamentally different effects of mating rates on reproduction success of the two sexes were later supported by Bateman with experiments on *Drosophila*. These experiments showed that males increase their reproductive output with every additional mating, while females will generally not benefit from mating with many males (polyandry) but from selecting a male that invests most in mating (Bateman 1948) and parental care (emphasized later by Trivers 1972).

However, many mating systems in various animal taxa deviate from the classical sex roles. Males may, for example, only mate with a single female (monogyny). In monogynous mating systems, maximal mating rates of females are higher than those of males (reviewed in Schneider and Fromhage 2010). Monogyny is predicted to evolve under strong male-male competition for the fertilisation of

females, which can be the result of a male-biased sex ratio (Fromhage et al. 2005). Combined with male mating strategies that protect their paternity with a single female, monogynous males can increase their reproductive success above the average in the population (Fromhage et al. 2005; Fromhage et al. 2008). Mating systems with low male mating rates and polyandrous females are taxonomically widespread, but have evolved independently in several spider species (Miller 2007). These species show an extreme sexual size-dimorphism with males being much smaller than females. Females are sexual cannibalistic and males have evolved remarkable adaptations such as self-sacrifice, life-long mate guarding and genital damage to secure the monopolisation of a female (Schneider and Fromhage 2010). While the evolution of male mating strategies and male interests are well understood in several monogynous mating systems (Elgar and Schneider 2004; Andrade and Kasumovic 2005; Schneider and Fromhage 2010), the evolutionary interests of females are still unclear.

In the following, I will concentrate on two main aspects of monogynous mating systems, which considerably differ from the classical mating systems and are therefore particularly interesting for behavioural research. First, I will focus on polyandry and potential genetic benefits that lead to the evolutionary significance and maintenance of polyandry in monogynous mating systems. Second, I will describe the frequency of male mate choice and why it might be highly adaptive for males to show some mate preferences in monogynous mating systems. As monogynous mating systems are highly pronounced in spiders, they are an ideal model organism in this research field.

Multiple mating is often combined with costs for females such as loss of time and energy (Watson et al. 1998), an increased risk of predation (Herberstein et al. 2002), infection (Poiani and Wilks 2000) and/or injuries (Blanckenhorn et al. 2002) and should therefore only evolve if the benefits of

polyandry outweigh these costs. In many species, females gain direct benefits that enhance their fitness through material resources provided by males (Arnqvist and Nilsson 2000). However, direct benefits associated with polyandry are often absent or small. In these cases, there is growing evidence that females profit from polyandry through genetic benefits that increase fitness of their offspring (Jennions and Petrie 2000; Tregenza and Wedell 2002; Zeh and Zeh 2003; Simmons 2005). Many hypotheses have been suggested to explain how females gain genetic benefits by mating multiply. The most promising explanation is that polyandry promotes the occurrence of sperm from multiple males at the site of fertilisation, which enables females to bias paternity post-copulatorily towards males with certain genotypes. This can happen indirectly through sperm competition or directly through cryptic female choice (Eberhard 1996; Simmons 2005). Males of a preferred genotype can either be of high genetic quality (good gene hypothesis) or most compatible with the female genotype (genetic compatibility hypothesis) (Jennions and Petrie 2000; Neff and Pitcher 2005; Simmons 2005). The number of studies showing evidence for post-copulatory cryptic female choice is rare, but most of them revealed that females discriminate against incompatible males, often in the context of avoiding or reducing the costs of inbreeding (Tregenza and Wedell 2002; Bretman et al. 2004; Bretman et al. 2009; Tuni et al. 2013). Inbreeding generally results in reduced offspring fitness, known as inbreeding depression, caused by the increase of homozygous deleterious alleles or the loss of heterozygosity (Charlesworth and Charlesworth 1987; Charlesworth and Willis 2009) and hence mechanisms to avoid or reduce matings between relatives are highly adaptive.

Such genetic benefits might be relevant for the maintenance of polyandry in monogynous mating systems of spiders. Here, most males provide no material resources and no paternal care, so that female choice will be based on genetic quality and enhance offspring fitness due to good genes and/or genetic compatibility. Females of entelegyne spiders exhibit a notable genital morphology that promotes the possibility for post-copulatory choice mechanisms (Eberhard 2004). Female spiders have paired genital openings that lead

to two separate sperm storage organs (spermathecae) via independent insemination ducts (Foelix 2011). Corresponding to females' paired genitals, males have two secondary copulatory organs (pedipalps) that they use to transfer sperm into the female spermathecae. Both female spermathecae can be filled by the same or different males in two separate copulation bouts (Uhl 2002; Nessler et al. 2007). The clear spatial separation of ejaculates may allow females to selectively use sperm and hence to control paternity. Recent studies demonstrated that polyandrous females in the spider genus *Argiope* possess means to cryptically bias paternity against sperm of siblings (Welke and Schneider 2009). This avoidance strategy is highly adaptive as inbreeding depression seems to affect these spiders (Welke 2012). Furthermore, pre-copulatory avoidance mechanisms for *Argiope* females appear to be absent and females are not choosy concerning their first mate. The latter was suggested to be a result of the risk of remaining unmated. However, females of some species in the genus *Argiope* are very aggressive and attack males already after the first copulation, which prevents the male from re-mating and consequently monopolisation if the female attacks successfully (Fromhage et al. 2003). Here, males monopolise females by damaging their pedipalps during copulation and leave a broken-off genital part as a mating plug inside the female genital opening (Nessler et al. 2007). If males plug both spermathecae with broken genital parts, no subsequent male is able to successfully copulate with the female (Nessler et al. 2007; Schneider and Lesmono 2009). Thus, the cannibalistic attack enables females to secure sufficient sperm for fertilisation of her eggs, but keeps the second spermatheca available for another insemination, which provides the opportunity of post-copulatory choice towards genetically compatible males.

Different from classical sex roles in which females show a larger investment in offspring and consequently carry a higher cost of inbreeding than promiscuous males, resulting in stronger selection to avoid costs, males in monogynous systems suffer similar fitness costs from inbreeding and should also have the interest of minimising costs through incompatibility. Indeed, males that mate with a sibling will copulate shorter and maximise

their chances to escape sexual cannibalism to search for a more compatible mate (Welke and Schneider 2010). Furthermore, males frequently reject virgin females for no obvious phenotypic reasons (Schulte et al. 2010), suggesting that males reject virgin females on the base of their genetic compatibility. Due to the above mentioned conditions, the orb-web spider genus *Argiope* is an excellent model for investigating whether the genetic compatibility hypothesis, in particular non-additive genetic benefits through the avoidance of inbreeding, can explain mating strategies of both sexes in mating systems with low mating rates.

Regarding the classical mating concept, monogynous mating systems do not only differ in the mating rates of the sexes, but males in this system often seem to be choosy and show mate preferences despite the absence of paternal care. In general, mate choice is expected when the benefits of choosing exceed the costs of choice and the relative costs of breeding for each sex is an elementary factor that determines which sex will be more selective (Kokko and Mappes 2005). In many cases the females invest more in each breeding event and should therefore be the choosy sex. However, males may also benefit from being choosy if there is variation in female quality and males are limited in their mating opportunities by high reproductive costs including mating effort (Bonduriansky 2001; Edward and Chapman 2011). Nevertheless, the benefits of choice may decrease if mate assessment is sequential and the rejection of a potential mate is attended with the risk of mating failures due to unpredictable mate availability (Barry and Kokko 2010). As benefits and costs of choice often vary depending on temporal and environmental conditions (e.g. population density, sex ratio, male-male competition), males should adapt their mate preferences according to the circumstances, as well as on their own internal state (Venner et al. 2010).

In many monogynous mating systems of spiders, females vary in quality and males show a high mating effort due to genital mutilation (Nessler et al. 2007; Uhl et al. 2007). Here, males that invest terminally into mating with a single female are expected to be choosy as their total reproductive success is defined by

the quality of this female. Female quality is associated with fecundity and mating status, which are prominent indicators of female reproductive value for males and that should influence males' investment into mating. Fecundity is generally predicted by female weight in spiders and males would benefit from choosing fecund females, as choosing a non-receptive female is accompanied by losses of time and energy. Several studies have demonstrated male preference for more fecund females (Danielson-Francois et al. 2002; Schulte et al. 2010; MacLeod and Andrade 2014). In entelegyne spiders, in which females can store sperm from different males in two separate spermathecae promoting the presence of sperm competition, female mating status should have also a strong selective effect on male mating preferences. Indeed, several studies revealed that males have a strong preference for virgin over mated females (Herberstein et al. 2002; Gaskett et al. 2004; Stoltz et al. 2007; Tuni and Berger-Tal 2012). This might be further adaptive as the existence of mating plugs leads to first male sperm priority in many species (Uhl et al. 2009). However, the existence of a mating plug in one of the two female genital openings does not prevent copulation into the other unused genital opening, so that males can still expect a 50% paternity share. Hence, mated females are still expected to be a suitable mating partner for males, particularly if the costs of mate search are high and the possibility to find another potential mate is unpredictable. Due to restricted male mating rates and a rapidly changing reproductive landscape over a short season (Zimmer et al. 2012), accompanied with varying mating behaviour, the orb-web spider genus *Argiope* is highly interesting for investigating the evolution of mate preferences in males. Furthermore, *Argiope* species differ in their mating strategies (mono- versus bigynous males) and whether or not these strategies are conditional, which make them ideal for comparative studies.

In my thesis, I combine field and laboratory work with genetic analyses to quantify the potential for inbreeding and inbreeding avoidance mechanisms (such as polyandry and trade-up mate choices) in the spider genus *Argiope*. Moreover, I investigate adaptive male mating strategies as a response to context- and state-dependent conditions.

## Study organisms

This thesis comprises experiments with three species of the orb-weaving spider genus *Argiope* (Araneae: Araneidae), which have a mating system with polyandrous females and monogynous males (mate with maximally a single female) or bigynous males (mate with maximally two females). This remarkable mating system and the associated adaptations have led to increased research examining the large variety of reproductive behaviours in this genus (Schneider and Andrade 2011). In all of these species, the males are much smaller than the females (sexual size-dimorphism) and are attacked by the female during mating (Herberstein et al. 2005b; Schneider et al. 2006), leading to different cannibalism rates among species. In each copulation, males empty one of their paired pedipalps into one of the female's paired genital openings. This mating pattern is particularly strict in the genus *Argiope*, in which the complex morphology of male genital apparatus together with the external female morphology only permits a fixed ipsilateral insemination pattern (Uhl et al. 2007), i.e. males can only use the right pedipalp to insert into a female's right genital opening and the left pedipalp into a female's left genital opening. Males always damage their genitals during mating (Nessler et al. 2007; Herberstein et al. 2012). This genital mutilation renders males functionally sterile after using both of their pedipalps and restricts them to two copulation opportunities in their lifetime. Males that survive the female's cannibalistic attack during the first copulation can decide to either mate again with the same female and to completely monopolise her by plugging both of her genital openings (monogyny) or to mate with a different female (bigyny) (Welke et al. 2012). All males inevitably die during their second copulation.

*Argiope* has a highly seasonal reproductive period and the mating season lasts about 3-4 weeks in *A. bruennichi* and up to two months in *A. keyserlingi* as well as *A. lobata*. Most males mature a few days earlier than females. Females are sedentary, while males build webs until their final moult and upon maturation they walk around to search for potential mating partners.

## Study aims

### Sexually selected compatibility

#### *Risk and costs of inbreeding*

In Chapter 1, I determine the natural risk and costs of inbreeding in four geographically separated populations of *Argiope bruennichi*. This species has recently extended its range from Southern Europe and Asia to the north of Europe (Guttmann 1979; Kumschick et al. 2011), which may lead to a genetic variation among these populations. As usually only a small number of individuals from the original population establish a new population, newly founded populations represent only a fraction of the gene pool of the source population (Mayr 1963) and will experience an increased risk of inbreeding, at least in the short term. However, as *A. bruennichi* seem to be successful dispersers, spiders can still expect an amount of genetically diverse individuals in the new populations. This predicts a scenario in which inbreeding risk through the probability of sibling encounters and costs of inbreeding in the form of inbreeding depression is present. In order to measure the individual risk of inbreeding, I sample egg sacs and individuals from four populations of likely different short-term settlement history within the recently colonised range of the species and assess the genetic diversity by using microsatellite markers. Furthermore, I assemble mating pairs derived from the same egg sac, from different egg sacs of the same population, or from two different populations and correlate the genetic distance of the mating partners with mating behaviour and hatching success to assess the individual costs of inbreeding. I expect differences in mating behaviour with increasing relatedness of the mating partners and expect genetic distance to be positively correlated with hatching success.

To measure the potential for individual inbreeding in a more precise way, it is useful to identify the genetic population substructure on a scale relevant for mating and distance covered by males during mate search. In chapter 2, I analyse the spatial genetic structure at a fine scale by spatially structured sampling of immature spiders from three *A. bruennichi* populations and using microsatellite markers.



Although *A. bruennichi* are successful dispersers, it can be safely assumed that at least some spiderlings remain in the proximity of their natal egg sac to take advantage of the proven quality of the natal site, creating a population substructure with patches of closely related individuals. Hence, I expect to find a population substructure with neighbouring individuals that are genetically more similar than distant individuals, leading to a risk of inbreeding. As this spatial pattern and the corresponding individual risk of inbreeding might change due to male mate search behaviour, I collect and genotype sub-adult females and adult males that are luring in their immediate vicinity within the same three populations to determine the probability that mature males guard genetically similar sub-adult females.

Finally, I test the existence of pre-copulatory inbreeding avoidance in males in a natural population of *A. bruennichi*. Previous studies have shown that males frequently reject virgin females for no obvious phenotypic reason (Schulte et al. 2010). I predict that males reject females based on their genetic compatibility. For that, I monitor mate acceptance or rejections in the field, genetically screen the mating partners, and assess the genetic distance between the male and female.

#### *Strategic multiple mating*

In chapter 3, I reveal that *A. bruennichi* females adapt their mating behaviour on the benefit of polyandry to avoid inbreeding. Females are sedentary and initially mate indiscriminately possibly to prevent remaining unmated. Mating multiply would give them the opportunity to cryptically bias paternity towards the superior male. However, it has been shown that females become unattractive to males after mating (Gaskett et al. 2004; Stoltz et al. 2007; Schulte et al. 2010; Tunj and Berger-Tal 2012) which might be a result of a variation in pheromone production. Female spiders are known to use silk-based or cuticle based sex pheromones to attract mates (Gaskett 2007) and the change in pheromone production might be a strategy of females to control mate attraction (Thomas 2011). If the costs of polyandry are high, mated females might benefit from stopping their pheromone production to avoid additional matings. On the

other hand, females might benefit from re-advertising receptivity after mating with an unsuitable male to receive a further mating with an ideally high-quality male. This gives them the opportunity to post-copulatorily bias paternity in favour of the superior male. If this is the case, I expect that mated females are more attractive for males if they had previously copulated with a sibling rather than with a non-sibling male. I use a silk-based choice experiment to test whether mated females strategically release sex pheromones depending on the quality of the first mate.

#### *Opportunistic mate-choice*

In chapter 4, I use *A. lobata* to test for the existence of trade-up mechanisms as a strategy to avoid inbreeding. According to the trade-up hypothesis, females should mate indiscriminately with the first male they encounter to ensure fertilisation of their eggs, but then should mate with a genetically superior mate than the previous mating partner in successive matings (Halliday 1983; Jennions and Petrie 2000). I produce an inbred and an outbred line and offer females from both lines two males of different relatedness in various combinations. As no pre-copulatory discrimination against siblings has been found when the spiders are virgin, I expect that both females and males will be more willing to accept a second mate if the second mating partner is genetically more compatible than the previous partner. I predict that the advantage of this trade-up strategy should be intensified with increasing inbreeding.

### **Male mating strategies**

#### *Context- and state-dependent mate choice*

Male mate choice in spiders may have evolved due to the high mating investment and the large variety of females during mating season. In *A. bruennichi*, the mating season lasts only for 3-4 weeks and exhibits a strong male-biased operational sex ratio (OSR) in the beginning of the season and a complete female-biased OSR in the end (Welke et al. 2012; Zimmer et al. 2012), because males invariably die after their second copulation due to sexual cannibalism or spontaneous death. Hence, males can expect a seasonal variation of male-male competition and mate availability in the course of the mating season. Depending on the timing and varying environmental conditions over the

season, males should adapt their mate choice decisions to maximise their reproductive success. In chapter 5, I use *A. bruennichi* males of different mating status and different age and quantify their mate choice decisions between females of different states and developmental stages in binary choice tests. Furthermore, I vary the competitive context in experimental trials. I expect high variation within male mating strategies due to the variability in female quality, male state and male-male competition.

#### *Mate choice in response to sperm competition*

As *Argiope* females prefer to mate multiply, sperm of different males frequently occur at the fertilisation site promoting the competition between ejaculates of males for the fertilisation of the female's ova. Hence, it should be a male interest to detect the risk and intensity of sperm competition and to adjust mating preferences to avoid it. Furthermore, as *Argiope* exhibit a fixed insemination pattern and males use genital parts to plug females' genital openings to prevent rival males from mating, males should also consider the interference from mating plugs in their mate choice. In the Australian spider *A. keyserlingi*, males were never observed to mate twice with the same female (Herberstein et al. 2005a) and hence face the risk of encountering a single-mated female which virgin copulatory duct does not match his unused pedipalp or a double-mated female with both copulatory openings plugged. In chapter 6, I use a silk-based choice experiment to test whether *A. keyserlingi* males of different mating status can distinguish between females that mated with one or two males and whether single-mated males discriminate between single-mated females with matched or unmatched virgin genital openings. I expect that males prefer single-mated over double-mated females but are unable to discriminate between compatible or incompatible single-mated females by using silk cues. Furthermore, I investigate whether males adapt their mating strategies to the intensity of sperm competition by providing males with virgin or mated females in their immediate vicinity. I expect that males follow a monogynous mating strategy if the available females are mated and carry a risk of sperm competition and unmatched virgin genital openings and a bigynous mating strategy if the available females are virgin.

#### Note

The individual chapters of this thesis should be comprehensible as they are, without need for reference to other sections. A certain degree of overlap in the descriptions and explanations given is therefore inevitable.

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## Chapter 1

### Rapid range expansion is not restricted by inbreeding in a sexually cannibalistic spider

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Few studies investigated whether rapid range expansion is associated with an individual's short-term fitness costs due to an increased risk of inbred mating at the front of expansion. In mating systems with low male mating rates both sexes share potential inbreeding costs and general mechanisms to avoid or reduce these costs are expected. The spider *Argiope bruennichi* expanded its range recently and we asked whether rapid settlement of new sites exposes individuals to a risk of inbreeding. We sampled four geographically separated subpopulations, genotyped individuals, arranged matings and monitored hatching success. Hatching success was lowest in egg-sacs derived from sibling pairs and highest in egg-sacs derived from among-population crosses, while within-population crosses were intermediate. This indicates that inbreeding might affect hatching success in the wild. Unlike expected, differential hatching success of within- and among-population crosses did not correlate with genetic distance of mating pairs. In contrast, we found high genetic diversity based on 16 microsatellite markers and a fragment of the mitochondrial COI gene in all populations. Our results suggest that even a very recent settlement secures the presence of genetically different mating partners. This leads to costs of inbreeding since the population is not inbred.

**Keywords:** *Argiope*, polyandry, inbreeding depression, microsatellite, genetic diversity

#### Introduction

Inbreeding, defined as the mating between two related individuals, increases the occurrence of homozygous deleterious alleles. The loss of heterozygosity leads to a decrease in the fitness of offspring, known as inbreeding depression (Charlesworth and Charlesworth 1987) although inbreeding may also be associated with benefits (Szulkin et al. 2013). Inbreeding depression has been reported for most taxa and has led to a variety of inbreeding-avoidance mechanisms (Pusey and Wolf 1996) e.g. by sex differences in dispersal (Pusey 1987; Perrin and Mazalov 1999; Bilde et al. 2005), in life-history (Clarke et al. 2001; Bukowski and Aviles 2002), or by mate choice (Tregenza and Wedell 2000). The latter requires a kin-recognition mechanism (but see Yasui 1998) and can occur before but also after mating (Penn and Potts 1999; Lihoreau et al. 2007). Post-mating sexual selection requires multiple mating by females which increases copulation costs that should be offset, at least in part, by benefits (Zeh and Zeh 1996). Such benefits are particularly enigmatic if they are only of an indirect nature (Jennions and Petrie

2000). Indeed, avoidance or reduction of inbreeding costs through post-copulatory mate choice have been identified as a major benefit of female multiple mating in several taxa, such as house mice (Firman and Simmons 2008), birds (Birkhead and Moller 1995), field crickets (Tregenza and Wedell 2002; Bretman et al. 2004; Bretman et al. 2009), and spiders (Welke and Schneider 2009).

In mating systems with classical sex roles (unselective males maximise fitness by increasing mating rates while reproductive success of females does not increase linearly with the number of mates (Bateman 1948)), females show a larger investment per offspring (Parker 1979; Smith 1979) and suffer more from inbreeding through the loss of their individual fitness than males that only invested some sperm. Thus, selection to avoid inbreeding in the context of individual fitness costs should act particularly strong on females. However, in mating systems characterised by low male mating rates, males suffer similar fitness costs from inbreeding as females and avoidance of inbreeding should also be favoured in males. Indeed, when male mating rates are lower than female mating rates, selection should act more

strongly on males than on females, particularly when polyandrous females possess means of cryptic female choice. These conditions are met in monogynous or bigynous mating systems, which are especially common in spiders (Herberstein et al. 2005; Fromhage et al. 2008; Schneider and Fromhage 2010; Welke et al. 2012). Males in such mating systems restrict themselves to mating with a single or maximally two females while females appear to favour multiple mating (Miller 2007; Schneider and Fromhage 2010). It has been suggested that females oppose monopolisation by a single male through post-copulatory discrimination against less compatible males and there is some evidence that females cryptically discriminate against the sperm of related males (Welke and Schneider 2009). However, to date no study has directly measured natural risks and costs of inbreeding for an individual in such mating systems.

Inbreeding is particularly likely if a small number of individuals split off from the original population and establish a new population representing only a fraction of the gene pool of the source population (Mayr 1963). Furthermore, the co-settlement of siblings may promote the risk of inbreeding in the newly founded population. Analogous to the classical scenarios of founding populations and bottlenecks, although only short-term, species that actively expand their range will likely experience a decrease in genetic diversity at the forefront of range expansion in comparison to populations in the centre of a species' range (Eckert et al. 2008). This may result in an increased risk of inbreeding at least in the short term. Individuals that reproduce in a new patch may be faced with a reduced choice of mating partners that are perhaps even siblings. The lack of compatible mating partners can entail fitness costs as even one generation of inbreeding can lead to drastic fitness losses of the offspring, e.g. in terms of reduced competitive fertilisation success as reported for male *Telegryllus oceanicus* (Simmons 2011) or reduced adult lifespan in the spider *Argiope australis* (Welke and Schneider unpublished data). However, some species are tolerant to short-term inbreeding as for example *Stegodyphus lineatus* (Bilde et al. 2005), *Oedothorax apicatus* (Bilde et al. 2007) and *Anelosimus cf. jucundus* (Aviles and Bukowski 2006). The degree of inbreeding depression can

vary depending on the size and age of the mating population (Jamieson 2011), as well as the potentially involved purging of deleterious recessive alleles (Barrett and Charlesworth 1991; Crnokrak and Barrett 2002). Generally, species that are successful colonisers are expected to show some tolerance towards the negative effects of inbreeding (Purcell et al. 2012) or a dispersal mode that does ensure genetic diversity even in newly founded sites.

Here, we use the spider *Argiope bruennichi* (Araneae) that unites a mono- and bigynous mating system and has recently extended its range from southern Europe and Asia to Northern Europe (Kumschick et al. 2011). The rapid colonisation implies that *A. bruennichi* can be considered a successful disperser. In combination with the observation that the species has started its range expansion from a large source population, it is likely that newly established populations even by small numbers of individuals encompass some genetic variation. *A. bruennichi* disperses aurally by ballooning and bridging to move within habitats. This passive mode of dispersal, particularly ballooning, entails a large component of chance as individuals can only influence direction by selecting certain wind conditions to fly (Suter 1991; Bonte et al. 2003). The expansion would likely occur through small numbers of individuals establishing new populations and as new meadows are colonised, individual females can expect high reproductive success. *A. bruennichi* spiderlings hatch simultaneously from large clutches after winter and likely disperse in groups from the same brood when conditions are favourable. This will lead to a situation in which many siblings from a single female are likely present in a meadow that also contains other families. Spiderlings may disperse short or long distances. This scenario creates both, inbreeding risk as siblings encounter one another and costs of inbreeding (note that costs of inbreeding require a population that is not inbred). Such a scenario matches data derived from mating experiments and field observations that demonstrated selection to avoid the costs of inbreeding (Welke and Schneider 2009). Hence, we predict that genetic diversity is present in small recently colonised meadows but that sibling matings will occur. As a consequence, we predict the presence of inbreeding depression

from within-population matings, which should be absent in among-population matings. Hence, we expect a larger variation in hatching success resulting from the former matches in comparison from the latter ones and we expect this to be matched by the occurrence of sibling matches within populations.

We collected *A. bruennichi* egg-sacs and juveniles from four similar sized populations located near the northern edges of the species range. We assessed genetic diversity by analysing 16 microsatellite loci and a part of the mitochondrial COI gene. Furthermore, we assembled mating pairs that stemmed from the same egg-sac, from different egg-sacs of the same population or from two different populations and correlated the genetic distance of the mating partners with mating behaviour and hatching success. We predicted differences in mating behaviour with increasing relatedness of the mating partners and expected genetic distance to be positively correlated with hatching success.

While the sampled populations are all located within the recently colonised range of the species, they likely differ in their short-term settlement history in that they may have been populated early in the invasion process or in recent years.

## Material and Methods

### Study species

*Argiope bruennichi* (Scopoli 1772) did not occur in Northern Europe until the beginning of the 20<sup>th</sup> century with the exception of an isolated group around Berlin (Krehenwinkel and Tautz 2013). It expanded its range since around 1930 (Guttmann 1979; Kumschick et al. 2011) and colonised Northern Germany including the region around Hamburg since 1975 (Guttmann 1979). Today, these spiders are very common on meadows all over Northern Europe and can occur in densities of about 3 webs / m<sup>2</sup> (Zimmer SM, personal observation).

As typical of entelegyne spiders, *A. bruennichi* possess paired mating organs. Females have two copulatory openings that are connected by two ducts to independent sperm storage organs (spermathecae) (Foelix 2011). The two spermathecae can be filled separately by the same or two and rarely three males

(Nessler et al. 2007; Uhl et al. 2007). Males have two secondary copulatory organs, the pedipalps, which they use to transfer their sperm. Because males damage their pedipalps during copulation, they can use both of them only once. The damaged genital part acts as a plug in the female's genital opening and is very effective in preventing rivals to mate into the same opening. This mechanism limits a female's mating rate (Nessler et al. 2007).

Females show a highly aggressive mating behaviour. All females attack males during copulation and 80 % of males are cannibalised by the female after mating (Schneider et al. 2006). These males have used only one of their paired pedipalps. Males that survive their first copulation may return and inseminate the second spermatheca of the same female or they may leave and search for a second mating partner (Welke et al. 2012). All males inevitably die during their second copulation which can be found in other *Argiope* species as well (Sasaki and Iwahashi 1995; Foellmer and Fairbairn 2003).

### Study Animals

We collected egg-sacs and juveniles from four geographically separated populations in the northern part of Germany (distance between population locations range between 42 and 148 km; Pevestorf (53°03'40.69" N, 11°28'24.59" E), Quarrendorf (53°15'51.81" N, 10°01'30.74" E), Buxtehude (53°27'10.37" N, 9°40'23.67" E), and Hamburg-Moorfleet (HH-Moorfleet; 53°30'37.30" N, 10°6'1.60" E)) between the end of April and the beginning of June 2010. There were no specific permissions required for these locations and the sampling did not involve endangered or protected species.

The collected egg-sacs were produced in 2009 and had overwintered. Several hundred spiderlings hatch out of the same egg-sac (Crome and Crome 1961) and can hence be unambiguously labelled as siblings, although females may mate with two different males that share paternity so that spiderlings from the same egg-sac could be full or half-siblings (Zimmer et al. 2012). The relatedness of juveniles could not be determined, so that these animals could not be used for sibling matings (see below).

387 individuals were raised from eggs in the laboratory until they reached adulthood. Each

spider was individually labelled so that it was known from which population and from which egg-sac it derived. Males were kept in individual 250 ml plastic cups, whereas subadult females were housed in 330 ml plastic cups and were transferred in individual Perspex frames (36 × 36 × 6 cm) after they moulted to maturity. Mating trials were conducted in the frames, where females built their typical orb-webs. All spiders were sprayed with water five days a week. Males were fed with approx. 15 *Drosophila* spec., subadult and adult females with three *Calliphora* spec. on two days a week. After individuals' final moult, both females and males were weighed on an electronic balance (Mettler Toledo AB54-S) to the nearest 0.001 mg. All males and females used in the mating experiments were frozen at - 80°C and preserved for genetic analyses (see below). Males were preserved after a single copulation and females were kept in the laboratory to produce egg-sacs until they died a natural death.

### Mating experiments

We experimentally staged and closely observed matings between siblings (N=32), between non-siblings from the same population (N=45) and between non-siblings from different populations (N=62). Egg-sacs were collected from two populations (Buxtehude and HH-Moorfleet) so that we derived 15 maternal lines (8 from the population Buxtehude, 7 from the population HH-Moorfleet). Females and males from these matrilineages were randomly assigned to one of the three mating trials. Spiders that were collected as juveniles were only used in the treatment where we arranged matings between different populations. 32 females from population HH-Moorfleet and Buxtehude were paired with males from the same family (sibling pairs; hatched from the same egg-sac); 45 females from population HH-Moorfleet and Buxtehude were paired with males from the same population that hatched out of a different egg-sac and 62 were mated to males that originated from different populations (HH-Moorfleet, Buxtehude, Quarrendorf, Pevestorf). Each mating pair was allowed a single copulation. Mating trials began by introducing the adult male into the frame threads of the female's web. Trials were terminated after the first copulation. If no mating occurred until one hour had passed, a new male was introduced to

the female. A female was presented with a maximum of three males. It never happened that a female was not mated after introducing the third male. During every mating trial, we noted the times of male's first contact with the web and the female, the beginning and duration of courtship and copulation, the insemination duct the male copulated into and the occurrence of sexual cannibalism or male escape from a female attack.

### Hatching success

Mated females were transferred from the frame into 500 ml plastic cups where they built their egg-sacs. We obtained egg-sacs from 95 females, each of which produced  $3.37 \pm 0.18$  egg-sacs on average. All egg-sacs were weighed on the day of their construction and were visually inspected. Some egg-sacs were damaged or not completed. We selected all intact egg-sacs and left them to hatch. After the young had hatched from the eggs, egg-sacs were preserved and all eggs and spiderlings were counted under the microscope. Hatching success of all intact egg-sacs was determined by the following calculation:  $\text{total number of spiderlings} / ((\text{total number of eggs} + \text{spiderlings}) / 100)$ .

### Microsatellite analysis and mitochondrial sequencing

We used microsatellite typing to estimate genetic distance (measured as the individual proportion of shared alleles; POA) between individuals within and among the four populations of *A. bruennichi*. We were able to determine genetic distances in seven sibling pairs, 11 within-population pairs and in 31 among-population pairs.

For this, we extracted DNA with the 5 PRIME ArchivePure DNA Kit according to the manufacturer's protocol (5 PRIME, Hamburg, Germany).

We genotyped our specimens for a set of 16 previously developed microsatellite loci for *A. bruennichi* (Krehenwinkel and Tautz 2013). PCR amplification was performed according to the Qiagen Multiplex PCR Kit Protocol (see Qiagen, Hilden, Germany). We used ABI ROX size standard as size standard. Genotyping was performed on an Applied Biosystems 3730 DNA Analyzer. Microsatellite alleles were then called using GeneMapper 4.0 (Applied



Biosystems). Genetic distances (POSA) within and among populations, as well as the overall  $F_{ST}$  value and pair-wise  $F_{ST}$  values among populations were calculated using Microsatellite Analyser (MSA) 4.05 (Dieringer and Schlotterer 2003). Furthermore, we calculated heterozygosity of each individual and the allelic richness per population across the 16 microsatellite loci using MSA 4.05.

Due to the presence of null alleles (one or more alleles fail to amplify during PCR) for the microsatellite screened, detected with the software Microchecker 2.2.3 (Van Oosterhout et al. 2004), we sequenced also a 1200 bp fragment of the mitochondrial COI gene as an additional marker. PCR and sequencing conditions are described in (Krehenwinkel and Tautz 2013). Sequences were edited using CodonCode Aligner (CodonCode Corporation, Centerville, USA) and aligned using ClustalW with default settings implemented in MEGA 4.0 (Tamura et al. 2007). Genetic Diversity (nucleotide and haplotype diversity) of the four populations was then calculated using DnaSP 5.10.1 (Librado and Rozas 2009).

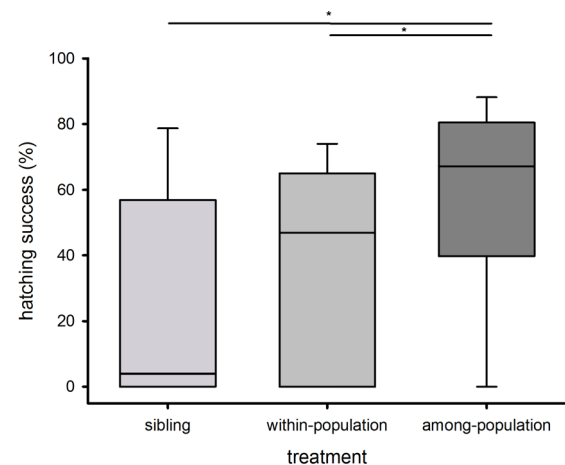
### Statistics

Most data were analysed with the statistical program JMP 7.0.2. Non-normally distributed data (and residuals) were analysed with the non-parametric Kruskal-Wallis test. Significant differences between groups were specified with the Dunn test (Zar 1996). Tests of equal variances were performed with the Brown-Forsythe test. Linear or logistic regressions were used to test the influence of genetic distances on mating behaviour and hatching success. A multiple regression was used to test the influence of female's and male's heterozygosity on the hatching success of their offspring. All tests are indicated with the results. Descriptive statistics are given as mean  $\pm$  standard error (SE). Sample sizes may differ between analyses due to missing data. Data are archived in Dryad: doi:10.5061/dryad.1np06.

## Results

### Hatching success

After a period of incubation, egg-sacs were opened and unhatched eggs and spiderlings were counted to determine hatching success. Hatching success was highly variable in all three treatments. As expected, the average hatching rate was lowest for sibling matings ( $28.18\% \pm 6.9$ ; median=3.95, N=21), followed by within-population matings ( $40.63\% \pm 5.27$ ; median=46.84, N=31) and was highest when the pair originated from different populations ( $57.0\% \pm 4.46$ ; median=67.15, N=43; Kruskal-Wallis test:  $\chi^2=13.12$ ,  $P=0.0014$ ; Figure 1 and Table 1). Multiple comparisons showed a significant difference of among-population and sibling groups (Dunn test;  $P<0.01$ ) as well as the among-population and within-population groups (Dunn test;  $P<0.05$ ); but comparisons between sibling groups and within-population groups were not statistically significant (Dunn test;  $P>0.5$ ; Figure 1). Variances in hatching success did not differ significantly between within-population and among-population matings (Brown-Forsythe test:  $F=0.16$ ,  $P=0.69$ ).



**Figure 1. Hatching success (%) of the three mating treatments (sibling, within-population and among-population pairs).** Hatching success was lowest for sibling matings (light grey), followed by within-population matings (grey) and was highest for among-population matings (dark grey). Box plots show the quartiles (box limits), the 10<sup>th</sup> and 90<sup>th</sup> percentiles (error bars) and the median (line). Statistically significant differences are indicated (\*).

**Table 1.** Summarised results of the three mating treatments in *A. bruennichi* including averaged observed Heterozygosity (Ho) per female and male, averaged proportion of shared alleles (POSA) and averaged hatching success per mating pairs.

Treatment	Female Ho	Male Ho	POSA	Hatching success
sibling pairs	0.35 ± 0.04 *(N=8)	0.34 ± 0.04 *(N=9)	0.37 ± 0.05 *(N=7)	28.18 ± 6.9 *(N=21)
within-population pairs	0.36 ± 0.05 *(N=11)	0.33 ± 0.03 *(N=19)	0.61 ± 0.03 *(N=11)	40.63 ± 5.27 *(N=31)
among-population pairs	0.36 ± 0.02 *(N=33)	0.34 ± 0.02 *(N=36)	0.62 ± 0.02 *(N=31)	56.99 ± 4.46 *(N=43)

\* Sample sizes may differ between results due to missing data.

**Table 2.** Summarised results for 16 microsatellite loci of four geographic different *A. bruennichi* populations including number of individuals per population (N), observed (Ho) and expected (He) heterozygosity per population, averaged proportion of shared alleles (POSA) per population as well as allelic richness (number of alleles per locus).

Population	N	Ho / He	POSA	Allelic richness	COI Nucleotide diversity*	COI Haplotype diversity*	No. of Haplotypes*
Hamburg-Moorfleet	65	0.38 / 0.62	0.58 ± 0.003	6.1	0.00082	0.61299	5
Buxtehude	67	0.31 / 0.61	0.57 ± 0.003	5.9	0.0015	0.70227	6
Quarrendorf	29	0.33 / 0.6	0.59 ± 0.005	6.4	0.00113	0.68923	4
Pevestorf	31	0.36 / 0.56	0.53 ± 0.005	5.6	0.00099	0.71077	5

\* Nucleotide diversity and Haplotype diversity, as well as the number of Haplotypes of the four populations were calculated by the mitochondrial COI gene using DnaSP 5.10.1.

**Table 3.** Pair-wise  $F_{ST}$  -values (below diagonal) and the p-values (determined by permutation; above diagonal) for the four *A. bruennichi* populations based on 16 microsatellite loci.

	HH-Moorfleet	Buxtehude	Quarrendorf	Pevestorf
HH-Moorfleet		0.0001	0.0028	0.0001
Buxtehude	0.060975		0.0001	0.0001
Quarrendorf	0.025936	0.0401		0.0017
Pevestorf	0.056521	0.069954	0.021097	

### Genetic differences within and between source populations

The variability of all 16 microsatellite loci was high in all four source populations with a range from 5.6 to 6.4 numbers of alleles per locus referred to as allelic richness (Table 2).

On average, all populations had a genetic distance between 0.5 and 0.6 (see Table 2). Comparison of the allelic richness among the four different populations across the 16 microsatellite loci did not reveal significant differences either (Kruskal-Wallis test:  $\chi^2=0.65$ ,  $P=0.89$ ,  $N=64$ ). The observed heterozygosity of the four populations ranged from 0.31 to 0.38

and was much lower than the expected heterozygosity (range from 0.59 to 0.62; Table 2). Furthermore, the nucleotide diversity (range from 0.0008 to 0.0015) as well as the haplotype diversity (range from 0.61 to 0.71) of the four populations calculated by the mitochondrial COI data set showed similar genetic diversities within the four populations (Table 2). The number of haplotypes of the four populations ranged from 4 to 6 (Table 2). The overall  $F_{ST}$  value showed a moderate, but significant differentiation (0.052;  $P=0.0001$ ) and differentiation between all population pairs were significant (Table 3).

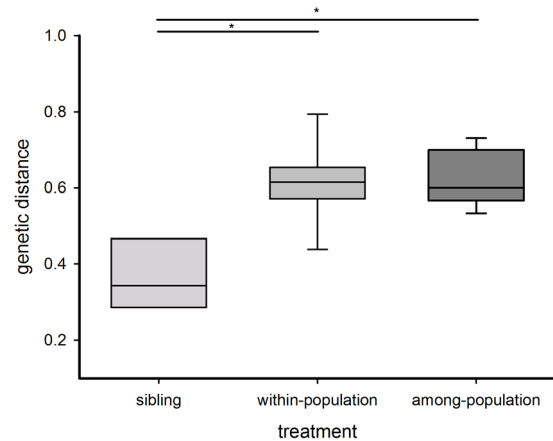
### Genetic composition of pairs and mating behaviour

We pooled all mating pairs regardless of their origin and tested whether the number of shared alleles between female and male of a mating pair correlated with components of their mating behaviour. The duration of copulation (linear regression:  $F_{1,47}=0.02$ ,  $r^2=0.0004$ ,  $P=0.9$ ) and the frequency of cannibalism (logistic regression:  $\chi^2=1.85$ ,  $P=0.17$ ,  $N=49$ ) were independent of the genetic distance between the mating partners. Furthermore, the genetic distance between a male and a female did not affect the time required until copulation occurred (linear regression:  $F_{1,47}=0.02$ ,  $r^2=0.0004$ ,  $P=0.89$ ).

### Genetic composition of pairs and hatching success

Comparing genetic distances among the three mating treatments, we expected to find the lowest genetic distance in sibling pairs, closely followed by a part of the within-population pairs while we expected the largest genetic distance in among-population pairs. A Kruskal-Wallis test showed significant differences of genetic distances between the three treatments ( $\chi^2=15.0$ ,  $P=0.0006$ ,  $N=49$ ). However, we did not detect significant differences between unrelated pairs derived from the same ( $0.61 \pm 0.03$ ) or from different populations ( $0.62 \pm 0.02$ ; Dunn test;  $P>0.2$ ; Figure 2) while as expected, siblings had the lowest genetic distance ( $0.37 \pm 0.05$ ; see Table 1) and significantly differed from the other two groups (Dunn test;  $P<0.001$ ). Variances in genetic distance did not differ significantly between the within-population and among-population groups (Brown-Forsythe test:  $F=0.02$ ,  $P=0.88$ ).

Using all mating pairs, the genetic distance between female and male of each pair did not significantly predict hatching success of their egg-sacs (linear regression:  $F_{1,32}=1.28$ ,  $r^2=0.04$ ,  $P=0.27$ ; Table 1). A multiple regression with the observed heterozygosity of female ( $F_{1,33}=-0.55$ ,  $P=0.58$ ) and male of each pair ( $F_{1,33}=0.79$ ,  $P=0.44$ ) revealed no significant association with hatching success either (multiple regression:  $F_{2,31}=0.37$ ,  $r^2=0.02$ ,  $P=0.69$ ).



**Figure 2.** Genetic distance (POSA) of the three mating treatments (sibling, within-population and among-population pairs) measured as individual proportion of shared alleles. Box plots show the quartiles (box limits), the 10<sup>th</sup> and 90<sup>th</sup> percentiles (error bars) and the median (line). Statistically significant differences are indicated (\*).

### Discussion

In experimental mating trials, we found the lowest hatching success in egg-sacs from pairs derived from the same brood and the highest hatching success when members of a pair came from different populations, while hatching success was intermediate for pairs of the same population. We genotyped each individual using 16 polymorphic microsatellite markers and expected that the presumed increase of genetic distance between the above groups of mating pairs would be mirrored in estimated proportion of shared alleles. However, while our measures of genetic distance provided expected estimates for siblings, we neither detected differences in genetic diversity between our study populations nor could we relate reduced hatching success in clutches derived from within-population matings to genetic distance between mating pairs. There are two possible explanations for the inconclusive mismatch between genetic and reproductive data. Either reduced hatching success in within-population matings was not caused by inbreeding depression or our genetic markers alone were not appropriate to detect relatedness between pairs.

The low hatching success of egg-sacs from sibling pairs strongly suggests that the species would suffer from inbreeding depression if sibling matings did occur. Studies of other

species found much lower costs of sibling matings in terms of fitness traits such as hatching success, fecundity and survival (Bilde et al. 2005; Bilde et al. 2007) and significant negative effects were apparent after only three generations of inbreeding. It was suggested that spiders might show a high tolerance towards inbreeding, perhaps as an adaptation to cope with a relatively high incidence of sibling matings (Bilde et al. 2005). Our data imply a comparatively low tolerance to inbreeding in *A. bruennichi*, but also a low risk of inbreeding even in small, recently founded populations. Genetic diversity was high in all populations and was probably even underestimated as the sampling mostly occurred before a possible ballooning event.

Within-population matings resulted in an intermediate hatching success with a very high variation and the variation in genetic distance was also highest in this group. This corroborates our predicted scenario and may suggest that some pairings were distinctly less profitable than others while the majority matched well. By coincidence, the majority of pairings in this treatment may have used offspring from unrelated females. The high variance may tentatively suggest that there is a possibility of less compatible matings if spiders stayed close to their birth site. The design of this study may have not been sufficient to detect the actual probability of sibling matings. It is possible that such matings can only be estimated by investigating small-scale spatial patterning of individuals as it has been measured in e.g. insects (Hardy et al. 2008; Bretman et al. 2011). To date, we have no data on the within-population sub-structuring on a scale relevant for mating and distance covered by males during mate search in *A. bruennichi*. Therefore, we cannot accurately estimate the probability for individuals of encountering a sibling.

Generally, a loss in genetic variation would be expected in any species that colonises new habitats as most dispersal mechanisms will result in a small number of individuals that found new populations and hence only represent a subset of the genetic variation of the source population (Mayr 1963). Spiders lay their eggs in large clutches and egg-sacs of *A. bruennichi* contain several hundreds of eggs (Crome and Crome 1961). In species with an overwintering period such as *A. bruennichi*, all

egg-sacs in a population hatch very synchronously regardless of when they were produced (Schaefer 1977). The common dispersal mode after hatching in spiders is ballooning, which means that the animal releases a thread of silk until it is uplifted by thermic or wind (Bell et al. 2005). This mode of travelling is generally restricted to very small spiders and is risky since the spider has very limited options to control where it will be going (Suter 1991; Bonte et al. 2003). Hence one might expect that at least a proportion of hatchlings remain at their natal site, which has proven to be of sufficient quality. These spiders may disperse by walking or bridging and settle nearby, causing a population substructure with patches of individuals that are closely related. Such a pattern has been found in the eresid spiders *Stegodyphus lineatus* (Bilde et al. 2005) and *S. tentoriicola* (Ruch et al. 2009), in which newly established nests are clustered around maternal sites. Unless there is sex-specific early dispersal, males may mature in the proximity of their sisters promoting inbreeding. In *S. lineatus*, males initially mate close to their birth site accepting a risk of inbreeding and then adopt a long distance mate search of higher risk (Bilde et al. 2005). Furthermore, a few spider females can produce a lot of offspring and quickly fill suitable web-sites at a location with her offspring. Depending on the degree of substructure and the probability of mating with a sibling, selection should favour kin-recognition mechanisms during mate choice if inbreeding is associated with more costs than benefits. However, generally rejecting related individuals as mating partners can be disadvantageous if the probability of finding a different mating partner is unpredictable. Female web-building spiders do not actively search for mates and face a risk of remaining unmated, hence they may benefit from accepting any male initially to secure fertilisation of her eggs leaving options for further copulations with preferred sires. Polyandry will then be in the female's interest because paternity could be biased towards the best mate (Zeh and Zeh 1997; Jennions and Petrie 2000). Post-copulatory choice has been demonstrated in several *Argiope* species (Elgar et al. 2000; Schneider and Lesmono 2009) and it was shown to be based on relatedness in *A. lobata* in which females cryptically favour

sperm from unrelated males (Welke and Schneider 2009). Pre-copulatory recognition seems to be present as well, since siblings mate for shorter and have a lower rate of sexual cannibalism (Welke and Schneider 2010). Such a strategy enables males that survive their first copulation to leave and search for a better second mating opportunity (Fromhage and Schneider 2012). A trading-up mechanism, in which both, females and males, first mate indiscriminately to secure a sperm supply and then try to re-mate with a higher quality mate, appears to be relatively common in spiders (Welke and Schneider 2009).

While the above conditions largely apply for *A. bruennichi*, the high variation in all our samples strongly suggests that dispersal is very efficient in this species so that each patch of suitable habitat will soon be inhabited by a relatively large number of individuals from several origins (Krehenwinkel and Tautz 2013).

Even though our treatment of mating individuals that originated from the same population showed a reduced hatching success, this effect was not apparent in the genetic distance of the experimental pairs. Several authors suggest that a sufficient number of markers are required to detect inbreeding depression in natural populations (Pemberton 2004; Grueber et al. 2011). Even studies with a relatively large number of microsatellite loci (>20) gave poor evidence for inbreeding depression (Grueber et al. 2011). By using 16 polymorphic microsatellite loci we clearly detected the difference between siblings and non-siblings, but no differences within the latter group added to the notion that such measurements alone are not always appropriate to predict risks and costs of inbreeding. The reduced hatching success of within-population matches might have resulted from incompatibilities that are not detected using microsatellites.

Indeed, we found an amino acid change between Alanine and Threonine in the mitochondrial genome of several individuals. It seems that pairs in which females carry this mutation and mated with males from a different population exhibit a higher hatching success (unpublished data). The interaction between the mutation and genetic composition of mating pairs suggests more complex genetic interactions and might be one possible

explanation of the higher reproductive success of among-population pairs compared to the within-population pairs.

It remains an open question how relevant incompatible matings are in natural populations that may show a much larger intermixture of genotypes through long distance dispersal. The rapid range expansion of *A. bruennichi* suggests that they are potent ballooners although it is unclear whether all hatchlings of an egg-sac balloon or whether a proportion stays. Published accounts are inconsistent in this respect (Follner and Klarenberg 1995; Walter et al. 2005). One would expect that an obligate high-risk dispersal phase should be opposed by selection just as much as the opposite of no dispersal, which would facilitate inbreeding as well as kin competition.

As the calculation of heterozygosity based on the microsatellite data set revealed a conspicuous difference between observed and expected heterozygosity of the four populations that did not relate to the genetic distance data, the presence of null alleles was tested for each locus and was confirmed in some loci. Null alleles occur through a failure of amplification during PCR leading to an over-estimation of homozygotes. Therefore we chose the mitochondrial COI gene as an additional marker to better trace the genetic diversity of the four populations. However, a DnaSP analysis of the COI gene data confirmed the similar genetic diversity within the four populations. A comparison with other studies showed that null alleles seem to be widespread in spiders (Rutten et al. 2001; Bilde et al. 2009). This might be explained by enormous population sizes of spiders providing increased mutation opportunities that lead to changes in primer binding sites and consequently inaccurate sequencing with the designed microsatellite primers. Moreover, in *A. bruennichi* an admixture of different lineages occurs resulting in the introgression of Asian alleles in populations of Northern Europe (Krehenwinkel and Tautz 2013) that might lead to an excess of non-amplifying loci. Future studies on spiders that involve usage of microsatellite markers should be aware of a potentially high risk of null alleles.

In conclusion, our results show that sibling matings lead to severe inbreeding depression in

*A. bruennichi* spiders and that there should be strong selection for inbreeding avoidance. The genetic data suggest that active partner choice would be beneficial even in small and recently founded populations as the genetic diversity is high and consequently the probability of finding a compatible partner is generally high. However, reduced hatching success in pairings of spiders derived from egg-sacs of the same population was not mirrored in the genetic distance data. Incompatibilities other than those caused by inbreeding may be responsible for the reduced hatching success.

Due to the experimental exclusion of ballooning and missing data on small scale population sub-structuring in *A. bruennichi*, the probability for individuals of encountering siblings cannot yet be estimated accurately. Studies are under way to close this gap by identifying the genetic population structure of natural populations close to and during the mating season on a small spatial scale. Furthermore, future experiments are of interest to test whether *A. bruennichi* has evolved pre-copulatory avoidance mechanisms to prevent or at least reduce costs of inbreeding depression in the field. During field studies, we commonly observed that males reject virgin females in the field without any obvious reasons (Schulte et al. 2010; Zimmer et al. 2012). Incompatibilities that result in reduced hatching success might be a reason.

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## Chapter 2

### The strength of sexual selection on inbreeding avoidance in natural populations of a spider

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Inbreeding avoidance via mate choice is expected to evolve if the risk of inbreeding is high but avoidable, and there is sufficiently severe inbreeding depression. However, few studies have assessed the individual risk and potential to avoid inbreeding in natural populations, particularly in invertebrates. Females of the spider *Argiope bruennichi* are polyandrous and known to cryptically bias paternity towards non-siblings. Males are choosy and frequently reject virgin females for no obvious phenotypic reasons. To test whether male choosiness is based on genetic similarity and whether cryptic choice is a result of the risk of inbreeding in nature, we first analysed the fine-scale spatial genetic structure of three *A. bruennichi* populations. Secondly, we monitored mate acceptance or rejections in the field, genetically screened the mating partners and assessed the genetic distance between the male and female. We used 16 polymorphic microsatellite loci to determine genetic distances and spatial autocorrelation analysis to investigate genetic population structure. We found no fine-scale genetic substructure, but siblings occurred as nearest neighbours in all three populations. The probability of sibling encounters differed between populations, but might overall be large enough to favour the evolution of inbreeding avoidance mechanisms in nature. Furthermore, the data revealed no correlation between genetic similarity and male rejection against some virgin females suggesting that the reason for male negative attitudes is not genetic similarity, but might possibly be due to other incompatibilities.

**Keywords:** polyandry, inbreeding depression, mating strategy, microsatellite, dispersal, genetic population structure

#### Introduction

Female multiple mating (polyandry) is a widespread, yet puzzling phenomenon as females of many species accept non-trivial costs of mating to gain more sperm than required to fertilise their eggs. Different kinds of benefits for females have been postulated to outweigh the costs. Females obtain direct benefits through polyandry in species in which males provide females with material benefits such as nutrient donations before or during mating, future paternal care and additional nest sites (Arnqvist and Nilsson 2000). In species without such benefits, females might profit from polyandry through genetic benefits that increase fitness of their offspring. Genetic benefits of multiple mating include trading up from poor-quality mates (Kempenaers et al. 1992; Bateman et al. 2001; Pitcher et al. 2003; Laloï et al. 2011), and increased diversity of offspring that reduces sibling competition or facilitates adaptation to environmental

changes (Ridley 1993; Yasui 1998, 2001; Fox and Rauter 2003). The existence of genetic benefits in the form of good genes that improve the performance of offspring is debated and recent meta-analyses suggest that such effects are weak at most (Slatyer et al. 2012; Mautz et al. 2013). However, less controversial are post-copulatory mechanisms of paternity bias that enable polyandrous females to reduce or even avoid negative effects through genetic incompatibilities mediated by selfish genetic elements or by the consequences of inbreeding (Jennions and Petrie 2000; Tregenza and Wedell 2000; Zeh and Zeh 2003; Simmons 2005; Wedell 2013).

The evolution of inbreeding avoidance mechanisms makes adaptive sense despite potential kin selected benefits (but see Kokko and Ots 2006) if inbred individuals can suffer a high reduction of fitness through the loss of heterozygosity and the increased impact of recessive deleterious alleles (Charlesworth and Charlesworth 1987; Keller and Waller 2002;

Charlesworth and Willis 2009). Selection on mechanisms of inbreeding avoidance after mating depends on the interplay of polyandry and paternity bias mechanisms towards unrelated mates. Females that mate multiply promote the occurrence of sperm from multiple males at the time of fertilisation that enable them to cryptically choose sperm of preferred high quality males post-copulatorily (Eberhard 1996). However, selection on post-copulatory, as well as pre-copulatory inbreeding avoidance adaptations can differ in strength between natural populations (Barrett and Charlesworth 1991; Crnokrak and Barrett 2002; Jennions et al. 2004). For example, selection on inbreeding avoidance might be relaxed in populations in which females rarely encounter sibling males, and stronger in populations with a higher risk of experiencing inbreeding which will depend on factors such as the relationship between immigration and emigration and density.

Until now, only a few studies have investigated whether animals mate multiply to avoid inbreeding (Birkhead and Moller 1995; Olsson et al. 1996; Zeh et al. 1998; Kraaijeveld-Smit et al. 2002; Mack et al. 2002; Tregenza and Wedell 2002; Garner and Schmidt 2003; Bretman et al. 2004; Simmons et al. 2006; Bilde et al. 2007; Firman and Simmons 2008; Bretman et al. 2009; Tuni et al. 2013). All these studies used species in which males have a higher potential maximal mating rate than females. In such mating systems, the costs of indiscriminate mating are lower for males than for females and females who are providing the larger parental investment suffer higher fitness costs through inbreeding than the other sex (Parker 1979; Smith 1979) and are hence under stronger selection to avoid these costs. This is fundamentally different in monogynous and/or bigynous mating systems in which males have similar or even lower maximal mating rates than females so that both sexes share the interest of minimising costs through incompatibility. Under such conditions, selection should favour means to decrease the probability that genetically related individuals meet each other e.g. through sex specific dispersal (Schiegg et al. 2006; Szulkin and Sheldon 2008). Such mechanisms are, however, mostly known for vertebrates and far less so for invertebrates. Perhaps as a consequence, it is in the latter where post-copulatory avoidance

or reduction of inbreeding costs has often been found. Surprisingly, only a handful of studies have assessed the individual risk of inbreeding in natural populations of invertebrates (Trontti et al. 2005; Hardy et al. 2008; Bretman et al. 2011).

Here we attempt to assess the individual risk of inbreeding in natural populations of the wasp spider *Argiope bruennichi*. The species has a monogynous mating system with a conditional option of bigyny for males (Fromhage and Schneider 2012; Welke et al. 2012) while females mate multiply although rarely more than twice (Zimmer et al. 2012). Females are not choosy about their first mate, but if they encounter a 2<sup>nd</sup> male that is not related, they will re-mate and then may store relatively more sperm from this male (Schneider and Lesmono 2009; Welke and Schneider 2009; Zimmer and Schneider, unpublished). Females possess paired genital openings that lead to two separate sperm-storage organs (spermathecae) (Eberhard 2004; Foelix 2011; Herberstein et al. 2011). Each spermatheca can be filled independently by sperm from a different male during two copulation bouts, which facilitates cryptic female choice for genetically compatible sperm and a paternity bias towards non-siblings. However, for a selection pressure on inbreeding avoidance to be relevant, the potential for inbreeding and the opportunity to avoid it must be present.

Males of *A. bruennichi* are choosy and frequently reject virgin females for unknown reasons (Schulte et al. 2010; Zimmer et al. 2012). In a comprehensive field study, male mate rejection could not be related to any measure of female phenotype (Schulte et al. 2010). These observations inspired the prediction that males reject virgin females on the base of their genetic compatibility. If a male is experimentally provided with a sibling as only mate, he will copulate very briefly, thereby maximising his chances to escape sexual cannibalism in order to continue searching for another mate for his terminal investment (Welke and Schneider 2010). These laboratory findings suggest that males can distinguish between related and unrelated females and make adaptive decisions. However, do these findings also apply in the field, and are the above adaptations a result of the risk of inbreeding in nature?

In order to quantify the probability of individual inbreeding and hence the potential of post-copulatory inbreeding avoidance, and to investigate the existence of male pre-copulatory inbreeding avoidance mechanisms, we sampled spiders of both sexes during different developmental stages from four geographically separated populations of the wasp spider *A. bruennichi*.

We expected to find a population structure that facilitates encounters of siblings during a mating season but that also provides the possibility to find more compatible mating partners. These prospects might vary among populations, promoting flexible strategies that accommodate the trade-off between choosiness and reduced mating success. Furthermore, the risk of individual inbreeding and consequently the potential for inbreeding avoidance might vary temporally depending on male mobility. Males mature earlier than females and leave their webs after maturation to actively search for females. At that time, they accrue in the vicinity of sub-adult females (Zimmer et al. 2012). Males can then decide to stay close to their natal website and guard the nearest female as shown for the spider *Stegodyphus lineatus* (Bilde et al. 2005) or to conduct a risky mate search to find females further away.

By spatially structured sampling of immature spiders from three *A. bruennichi* populations and determining their genetic structures using microsatellite markers, we tested the hypothesis that neighbouring individuals are genetically more similar than distant individuals. Furthermore, by collecting and genotyping sub-adult females and the adult males that are in their immediate vicinity within the same three populations, we assessed the probability that mature males guard genetically similar sub-adult females. Finally, to test whether males avoid inbreeding pre-copulatorily, we closely observed virgin females in a natural population at the beginning of the mating season. We noted male and female behaviour prior to mating and collected the visiting males after they had decided whether to leave the female or to mate with her. Visiting males and the corresponding females were genetically screened and compared to each other.

## Material and Methods

### Study species

The orb-web spider *Argiope bruennichi* (Scopoli 1772) spread from Southern Europe and Asia to the north of Europe since around 1930 (Guttmann 1979; Kumschick et al. 2011) and colonised sunny, open meadows with long grass and low vegetation (Bellmann 2006). They build typical large orb-webs in meadows often a little above the ground decorated with a stabilimentum (Bruce 2006). The mating season starts in mid-July and lasts only about three to four weeks. *A. bruennichi* males and females mature in July, whereby most males mature a few days earlier than females. Females start to produce their egg sacs a month after maturation. The spiderlings hatch out of the eggs after around four weeks but overwinter inside the egg sacs. They emerge in spring and initially use a communal hunting web. After 1-2 moults, they disperse to build their own webs at suitable places and grow to maturity until July. The males build webs until their final moult and upon maturation they rove around to search for potential mating partners. Instead, females are sedentary and occasionally change their web location but stay within a radius of around one meter (Zimmer et al. 2012).

*A. bruennichi* exhibits a strong sexual size dimorphism with much smaller males. They are sexually cannibalistic spiders, in which females cannibalise up to 80% of their mates during mating (Schneider et al. 2006). *A. bruennichi* males have two secondary copulatory organs, the pedipalps, which they use to transfer sperm (Foelix 2011). They damage their pedipalps during copulation and the broken-off pieces remain in the female's genital opening and thus function as a mating plug to prevent females from further mating (Nessler et al. 2007). However, males can use only one of their pedipalps at a time and thus the two separate sperm storage organs of females can only be filled in two separate copulation bouts.

### Sampling

Sampling was conducted to quantify the spatial genetic population structure within a spatial scale within walking distance of male spiders. For this, we collected individuals from three geographically separated populations (Hamburg-Billwerder (HH-Billwerder, 53°52'53.

79° N, 10°10'55.91" E), Pevestorf (53°06'46.77" N, 11°41'20.58" E) and Tostedt (53°28'97.09" N, 9°73'51.68" E) in Northern Germany. All three study sites are dry grasslands with patchy vegetation isolated by a road on the one side and a forest or industrial area on the other sides. High densities of grasshoppers were available as the main prey on all three meadows.

Sampling occurred twice. The first sampling took place shortly before the reproductive season in the end of June 2013 on three consecutive days, one day for each population. At that time, both sub-adult females and sub-adult males sit in their own webs. For the sampling, we marked with ropes a longitudinal transect of 1.5 meters width and at least 24 meters length that crossed the meadow and captured a representative part of the population. We established a grid by connecting the ropes that demarked the long sides with another rope every two meters. We sampled all individuals located within the transect. If the 24 meters length of the transect did not contain at least 50 spiders, the transect would be extended until the goal of sampling of at least 50 spiders was reached. We collected 59 individuals within the transect of 24 meters length from the meadow in HH-Billwerder, 66 individuals within the transect of 24 meters length from the meadow in Pevestorf (Lower Saxony) and 53 individuals within the transect of 26 meters length from the meadow in Tostedt (Lower Saxony). We GPS-mapped each web individually within the transect in each population and additionally noted the web location on a map based on the rope-grid. On the basis of GPS-mapping, we mapped and visualised distributions of individuals and computed the distances using Geographic Distance Matrix Generator (Ersts [Internet]). Subsequently, the collected individuals were genetically screened (see below).

The same three populations were revisited at the beginning of July 2013, shortly after the first sampling. At that time, adult virgin males have already moved around to search for potential mating partners and besiege the webs of sub-adult females. The second collections were done in sections of the meadows that were outside the previous transects and thus were unaffected by the previous sampling. We had marked the boundaries of transects with bamboo poles so that the sampled area was

easy to recognise. During the second sampling, we collected 20-21 sub-adult females with their male visitors from each of the three populations.

### **Spatial autocorrelation analysis**

The occurrence of genetic population structure on a small spatial scale was investigated using spatial autocorrelation analysis. This method tests whether genetic similarity between all possible pairs of individuals at one sample site depends on the geographic distance between them. If so, a pattern of isolation by distance exists that assumes a higher genetic similarity between nearby individuals (Barbujani 2000). Calculations were conducted using Spatial Genetic Software 1.0c (SGS) (Degen et al. 2001) by considering the mean number of alleles in common (NSC), over all 16 previously developed microsatellite loci, between pairs of samples to a given spatial distance class. Here, we chose five distance classes for the sample sites: 0-5, 5-10, 10-15, 15-20 and 20-25 m. Significant deviation of all values from a spatially random distribution of alleles were tested using a Monte Carlo permutation applied in SGS. The mean number of alleles in common over all pairs of individuals constitutes a reference value. Isolation by distance exists when individuals at short distances are significantly more similar than the reference value, whereas individuals at larger distances show insignificant values (Barbujani 2000).

### **Pre-copulatory inbreeding avoidance by males**

We conducted this field experiment on a meadow in the landscape conservation area Buxtehude (53°45'39.76" N, 9°67'43.68" E) in Lower Saxony (permission was given verbally by the owner) from 12<sup>th</sup> of July until 26<sup>th</sup> of July 2013. The study site is dry grassland with patchy vegetation including few low bushes surrounded by a forest and a small forest track. Shortly before the mating season, we marked the location of around 150 sub-adult females in the population with bamboo poles. Each day, we monitored the developmental status of each sub-adult female to forecast her date of final moult due to the swelling of her external genital structure (epigyne) (see Zimmer et al. 2012). In addition, we noted the number of males staying close to subadult females' webs or in their web each day. As soon as females

moulted to maturity, we gave them an individual number and continuously observed them until the first male visited them. We monitored if he rejected the female or mated with her, and any other behavioural interactions. Rejection was defined as follows: the male touched the web or the female and then left the web and walked away without any attempt to copulate. Thereafter, we collected the male and the female and froze them at  $-80^{\circ}\text{C}$  to determine their relatedness through microsatellite typing (see below). We assessed the genetic distance of 40 mating pairs (15 rejected, 25 accepted pairs). To analyse whether male's decision depended on genetic relatedness we used a logistic regression conducting in JMP 7.0.

### Microsatellite analysis

In order to determine the genetic similarity between pairs of individuals within the three sampling populations of *A. bruennichi* and the relatedness between potential mating pairs of the fourth population, we used microsatellite markers. Genetic divergence between individuals ('genetic distance') was measured as the individual proportion of shared alleles (POSA). Previous experiments revealed that siblings can be confidently detected with the markers and had a genetic distance value of around 0.3 while randomly sampled spiders from the same population had values around 0.5 or even larger (Zimmer et al. 2014). For microsatellite analyses, we extracted DNA with the 5 PRIME ArchivePure DNA Kit according to the manufacturer's protocol (5 PRIME, Hamburg, Germany). Our study species were genotyped for a set of 16 previously developed microsatellite loci for *A. bruennichi* (Krehenwinkel and Tautz 2013). PCR amplification was undertaken using the Qiagen Multiplex PCR Kit Protocol (see Qiagen, Hilden, Germany). We used ABI ROX size standard as

size standard. Genotyping was performed on an Applied Biosystems 3730 DNA Analyzer. Subsequently, microsatellite alleles were called using GeneMapper 4.0 (Applied Biosystems) and genetic distances between individuals were calculated using Microsatellite Analyser (MSA) 4.05 (Dieringer and Schlotterer 2003). The genetic distance ranges from zero to one. Smaller genetic distances (close to 0) indicate that individuals are genetically similar to each other, whereas greater genetic distances (close to 1) reveal less similarity between individuals.

## Results

### Spatial distance and relatedness

Spatial autocorrelation analysis of genetic structure in the three *A. bruennichi* populations showed no dependency between individual genetic distances (number of alleles in common, NSC) and spatial distances (Table 1) of juveniles. Individuals that were close together did not share more alleles than those that were far apart. Our data provided no evidence for a genetic population substructure.

During the second sampling, when adult virgin males were accumulating in the vicinity of sub-adult females that were close to their final moult, the mean genetic distance between females and visiting males was  $0.54 \pm 0.03$  (N=23) for the population Pevestorf,  $0.56 \pm 0.03$  (N=20) for the population HH-Billwerder and  $0.58 \pm 0.02$  (N=22) for the population Tostedt. Hence, the majority of males reached females that were genetically different with a genetic distance around 0.5. The probability of arriving at the web of a sister varied between 4.55 and 17.39%, suggesting that in some populations some siblings could potentially inbreed (Table 2).

**Table 1** Spatial autocorrelation analysis of fine-scale genetic structure in the three geographically separated *A. bruennichi* populations (Pevestorf, HH-Billwerder and Tostedt).

Population	Mean NSC over all pairs	Distance class limits (m)				
		0 - 5	5 - 10	10 - 15	15 - 20	20 - 25
Pevestorf	0.8690	0.8773 (n.s.)	0.8734 (n.s.)	0.8375 (n.s.)	0.8077 (n.s.)	
HH-Billwerder	0.7890	0.7973 (n.s.)	0.8089 (+*)	0.7825 (n.s.)	0.7524 (n.s.)	0.7038 (-*)
Tostedt	0.7420	0.7384 (n.s.)	0.7378 (n.s.)	0.7360 (n.s.)	0.7536 (n.s.)	0.7733 (n.s.)

Number of alleles in common (NSC) values indicate a mean differentiation of samples within each distance class. – = number of alleles in common significantly lower; + = number of alleles in common significantly greater than a mean differentiation over all distance classes. \* =  $P < 0.05$ ; n.s. = not significant.

**Table 2** Summarised results of first and second sampling from the three *A. bruennichi* populations (Pevestorf, HH-Billwerder and Tostedt).

Population	N (pairs of males and nearest female at 1. sampling)	Possibility that nearest female is a sister at 1. sampling	N (pairs at 2. sampling)	Possibility to have a brother in the vicinity/web
Pevestorf	24	12.5 %	23	17.39 %
HH-Billwerder	26	7.69 %	20	5 %
Tostedt	26	0 %	22	4.55 %

### Pre-copulatory inbreeding avoidance

Overall, we marked and observed 107 females that moulted to maturity during the field experiments. 28 of 107 (26.17%) females died, disappeared, or had no visitors during the observation and were not included into the data set. 62 of 107 females (57.94%) had at least one male visitor who mated with the female. 35 of the 62 accepted females (56.45%) had at least one male on her web during her final moult resulting in an opportunistic mating, meaning the male copulated with the defenceless female during her final moult (Foellmer and Fairbairn 2003; Uhl G, Zimmer SM, Renner D, Schneider JM, unpublished). It took males on average  $26.49 \pm 7.28$  min ( $N=33$ ) from the first contact to mating with the virgin female. 17 of 107 females (15.89%) had at least one male visitor who rejected the female and did not mate with her. Three of the 17 males (17.65%) who rejected females touched only the female's web and subsequently made their decision; the other 14 (82.35%) walked to the centre of the hub, touched the female and then left the web. On average, males rejected virgin females after  $17.42 \pm 10.06$  min ( $N=12$ ).

We excluded four males as outliers from the data due to their long latency before they made a decision ( $373 \pm 31.37$  min).

Contrary to expectation, males did not reject virgin females on the base of their genetic compatibility. Males that rejected virgin females were not genetically more similar to the females than males that accepted virgin females and mated with them (logistic regression:  $\chi^2_1=0.006$ ,  $P=0.94$ ,  $N=40$ ). The average genetic distance of rejected pairs was  $0.61 \pm 0.02$  ( $N=15$ ) and  $0.61 \pm 0.01$  ( $N=25$ ) for the accepted pairs.

### Discussion

Spatial autocorrelation results indicated that there was no fine-scale genetic substructure in three *A. bruennichi* populations; neighbouring spiders were not genetically more alike than average, so there were no isolation-by-distance patterns. Nevertheless, our results revealed a modest individual inbreeding risk within populations as relatives (genetic distance of around 0.3) did occasionally sit in the proximity and might encounter one another. However,



when we genotyped pairs upon encounter in the field we found that male mate rejection was not explained by genetic similarity (at least as measured), leaving the question what reasons can lead to mate rejection unresolved.

Such as most web-building spiders, *A. bruennichi* disperse by ballooning thereby accepting a high risk of ending up in unsuitable habitat and there is no consensus as to whether ballooning is obligate after hatching out of the egg sac (Follner and Klarenberg 1995) or not (Walter et al. 2005). Intuitively, some hatchlings should be short-distance dispersers that remain at their natal site of known quality (Walter et al. 2005). Since spiders simultaneously hatch out of egg sacs (which in *A. bruennichi* contain several 100 individuals), short-distance dispersers would create a population substructure with patches of closely related individuals as occurs in the subsocial spiders *Stegodyphus lineatus* (Bilde et al. 2005) and *S. tentoriicola* (Ruch et al. 2009), in which newly established nests are accumulated around maternal sites promoting inbreeding.

Population genetic studies on *A. bruennichi* suggest that this species colonises new habitats with a relatively large number of individuals from several origins (Krehenwinkel and Tautz 2013), and that diversity is high even in recently founded populations (Zimmer et al. 2014). Population genetic estimates do not necessarily consider the probability that individuals could still mate with relatives due to a fine-scale substructure. We observed high genetic diversity but still identified a modest risk of inbreeding within natural populations of *A. bruennichi*.

Settlement of juveniles in space may not be relevant for mating if sex specific dispersal later on will reduce the proximity of siblings. In the presence of a clustering of related individuals, male mate searching may cover a sufficient distance so they copulate with more distantly related females. Web-building spider females are relatively sedentary while males are mobile after maturation and actively search for receptive females (Foelix 2011). With our data set we can compare whether the spatial arrangement of mating partners is different from that of juveniles. We did not find a clustering of relatives among juveniles, and subsequently no difference in the spatial arrangement of the mating pairs. Males leave

their webs once mature and probably stay with the first female they encounter regardless of whether it is a sibling or not. The estimated probability that siblings would encounter each other varied between populations, was modest in two populations but reached a maximum of 17% in Pevestorf. Such a probability of inbreeding affecting two of 10 mating pairs appears high enough to justify selection on inbreeding avoidance mechanism particularly as inbreeding significantly reduces hatching success in *A. bruennichi* (Zimmer et al. 2014). A second generation of inbreeding leads to a drastic reduction in fitness by lowering adult lifespan in both sexes and causing smaller size in males in the congener *A. australis* (Welke 2012). However, both males and females reduce the costs of an incompatible first mating by actively inviting re-mating. Laboratory studies of several *Argiope* species have shown that polyandry and post-copulatory choice reduce the costs of inbreeding for females (Elgar et al. 2000; Schneider and Lesmono 2009). Females achieve this by controlling the number of sperm that they store from a second mate. Females of *A. lobata* have been shown to store similar numbers of sperm during first copulations with related or unrelated males, but sperm numbers in storage are significantly lower for second related males (Welke and Schneider 2009). Male *A. bruennichi* make use of the option to mate with two different females (bigynous strategy) if mated to a sister first; males copulate very briefly with sisters and thereby lower the risk of sexual cannibalism (Welke and Schneider 2010). Short copulations increase the chances for males to survive copulation and survivors search for a better second mating opportunity (Fromhage and Schneider 2012). Monogynous males copulate once or twice with the same female and thereby maximise paternity success with this female (Fromhage et al. 2005; Fromhage and Schneider 2012). Hence, in *Argiope*, both sexes do not reject closely related sexual partners when virgin and are selective only when further mating opportunities appear.

From the male perspective, pre-mating rejection of siblings seems to be more parsimonious than copulating with a sister and opting for bigyny. Even brief copulations entail a risk of sexual cannibalism and males may be better off by trying to find a compatible

partner right away. One explanation for the absence of rejection of sisters may be that some reproductive success even with a sister is still better than getting no mating at all. A risk of remaining unmated is often used as an explanation for trading-up strategies (Bleu et al. 2012). However, high densities and the genetic diversity in *A. bruennichi* do speak against this explanation. Furthermore, mate rejection does occur in the field as reported here and in a previous field study (Schulte et al. 2010) refuting the explanation of complete random mating. No phenotypic variables could be found to explain mate rejection (Schulte et al. 2010) nor did we find relatedness to be responsible for mate rejection. This leaves us to speculate that rejections might be due to genetic incompatibilities that were not detected using microsatellites. An alternative reason might be that rejected females were unsuitable mates because they were infected with parasites (Foelix 2011) or endosymbionts, which are quite common in spiders (Gunnarsson et al. 2009; Goodacre 2011), although unknown for *Argiope*. It is unclear how males collect information about female compatibility or parasite infection but chemical profiles are likely candidates. We know from previous studies that *Argiope* males can distinguish between females of different reproductive status using silk-based and airborne sex pheromones (Herberstein et al. 2002; Gaskett et al. 2004; Schulte et al. 2010). Sex pheromones may indeed code more information than just mating status, as shown in other spiders and insects (Johansson and Jones 2007). However, our current understanding of *Argiope* pheromones is limited.

Nevertheless, both sexes accept a risk of inbreeding as virgins and probably opt for multiple mating to reduce the negative impacts of inbreeding. However, the costs of inbreeding are not high enough to select for pre-copulatory mate rejection of unmated individuals.

To our knowledge our study is the first detailed microsatellite-based study of fine-scale (< 30m) spatial genetic structure in an orb-web spider. Although we used very polymorphic genetic markers and autocorrelation analysis, we failed to detect genetic substructure in *A. bruennichi* spiders. The reliability of our findings may be impaired by possible mistakes in measurements through

an inappropriate sampling scale or small sample sizes. Obtaining accurate results is dependent on the sampling scheme and on the selected distance intervals. Sampling along transects is a good strategy, because such small to large scales are considered to represent adequate pairs of individuals that are easy to map within a transect (Vekemans and Hardy 2004). However, sampling within a single transect in two dimensions might not precisely represent the distribution of individuals within populations and will miss or underestimate potential genetic structures (Vekemans and Hardy 2004). As *A. bruennichi* spiders seem to disperse or walk without direction, using transects in multiple dimensions may be better than single scales to assess spatial genetic structures in this species. Furthermore, to estimate genetic structures, the sample sizes must be large enough to ensure sufficient numbers of pair-wise comparisons in each selected distance class. Degen (2000) recommended a minimum of 30 pairs per distance class for analysis of spatial genetic population structures. As our analysis represents at least 30 pairs in all distance classes except for the 25 meter distance in one of the three populations (Pevestorf), we assume that our sample size was adequate to detect potential genetic structures in the three *A. bruennichi* populations. However, the use of suitable genetic markers has to be considered critically. Although the information content of microsatellites is expected to be sufficient for reliable estimates of spatial genetic structures, the number of genetic markers might not have been adequate: with more markers it is easier to detect weak spatial genetic structure (Vekemans and Hardy 2004). However, our markers were found useful to distinguish siblings from non-siblings (Zimmer et al. 2014) and since we were targeting the spatial distribution of siblings, the use of 16 genetic markers should have been sufficient.

In conclusion, we did not detect fine-scale genetic substructure in natural *A. bruennichi* populations but observed a modest probability of encountering a sibling. Hence, inbreeding is possible and this is already known to reduce hatching success and offspring fitness. The probability of sibling encounters differed among the populations and might overall be large enough to favour the evolution of inbreeding avoidance in nature but not large

enough to select against indiscriminate mating of virgin males and females. The presence and spatially random distribution of related individual spiders suggest that the dispersal mode does not lead to complete emigration. Rather an unknown proportion of spiders remains in their original habitat and intermingles with immigrating individuals leading to high local mix of diverse genotypes (Zimmer et al. 2014).

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## Chapter 3

### Strategic polyandry in the orb-web spider *Argiope bruennichi*

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Polyandry is taxonomically widespread and despite its costs it has been demonstrated that females gain direct and indirect benefits from mating multiply. In particular, genetic benefits in the form of good genes and/or genetic compatibility have been focus of recent studies. Polyandrous females are able to bias paternity towards preferred males post-copulatorily, which might be highly adaptive if sequential mate choice is combined with the risk of remaining unmated. In many spiders, females are sedentary and initially mate indiscriminately to avoid mating failures. Polyandry gives them the opportunity to trade-up towards superior males in successive matings. However, mated females of many spiders are known to become unattractive to males and it was suggested that this is the result of a different pheromone production. Whether this variation is a female strategy to control mate attraction is still unclear. Mated females might benefit from stopping their pheromone production to avoid additional matings if the costs of polyandry are high, but they might also benefit from re-advertising receptivity after mating with an unsuitable male to receive a further mating, ideally with a high-quality mate. By conducting a silk-based choice experiment, we tested whether mated females of the spider *Argiope bruennichi* strategically release sex pheromones depending on the quality of the first mate. We predicted that mated females are more attractive if they had copulated with a sibling rather than if they had mated with a non-sibling male. Our study demonstrated that females that had mated with a sibling seem to strategically continue advertising receptivity and thus attract further males. This suggests that females adapt mate attraction on the benefits of polyandry.

**Keywords:** polyandry, mate choice, inbreeding avoidance, cryptic female choice, sex pheromone

#### Introduction

Sexual selection arises from the differential reproductive success between individuals and occurs in two forms: male-male competition (intrasexual selection) and female mate choice (intersexual selection) (Darwin 1871). Many studies have concentrated only on sexual selection before copulation as females were assumed to be monogamous (Birkhead and Pizzari 2002). Due to the awareness that females of many taxa mate multiply, however, it was realised that sexual selection continues to exist after copulation via sperm competition and cryptic female choice (Birkhead and Pizzari 2002; Eberhard 2009; Birkhead 2010).

Polyandry promotes the occurrence of sperm from multiple males at the time of fertilisation favouring the evolution of behavioural, physiological and morphological male traits that enhance male's reproductive success towards competitive mates (Simmons

2001) and the opportunity of female-controlled paternity biases towards certain males (Eberhard 1996). Hence, polyandrous females that favour sperm of preferred high quality males post-copulatorily gain genetic benefits by increasing the fitness of their offspring due to good genes and/or genetic compatibility of the mating pair (Jennions and Petrie 2000; Simmons 2005). Post-copulatory female choice for genetically compatible males as part of inbreeding avoidance has been a focus of recent studies. Females in many taxa bias paternity towards unrelated males when they get the opportunity to mate with a sibling and a non-sibling male. Cryptic female choice for compatible sires has been demonstrated in birds (Birkhead and Moller 1995), house mice (Firman and Simmons 2008), crickets (Tregenza and Wedell 2002; Bretman et al. 2004; Bretman et al. 2009; Tuni et al. 2013) and spiders (Welke and Schneider 2009). Fitness consequences of favouring paternity by genetically compatible males is based e.g. on

the assumption that heterozygous individuals have a fitness advantage compared with homozygotes by limiting the negative expression of deleterious recessive alleles (Charlesworth and Charlesworth 1987; Keller and Waller 2002; Charlesworth and Willis 2009) and by increasing diversity in immunologically important genes (Penn and Potts 1999; Penn 2002; Milinski 2006; Schwensow et al. 2008).

Post-copulatory mate choice may be favoured by selection if sequential mate choice is combined with the risk of remaining unmated due to unpredictable mate availability (Kokko and Ots 2006). Here, polyandrous females have the opportunity to mate indiscriminately with the first male they encounter to secure fertilisation of their eggs. Once-mated females then re-mate if a subsequent male is of superior genetic quality (Halliday 1983; Jennions and Petrie 2000). Such a trade-up mechanism has been found in a number of species (Gabor and Halliday 1997; Bateman et al. 2001; Pitcher et al. 2003; Laloi et al. 2011). In many spiders, sedentary females that cannot actively search for a mate (Foelix 2011) are visited by mate-searching males sequentially during a temporary reproductive season (Zimmer et al. 2012). Females that are located at the edges of the population or that matured close to the end of the mating season may face a risk of remaining unmated. Hence, female spiders may benefit from polyandry because they need to match opportunity costs and the risk of collecting sperm of low quality. By mating multiply they have the option to cryptically bias paternity towards the superior male. However, females of many spider species are known to become unattractive to males after mating (Gaskett et al. 2004; Stoltz et al. 2007; Schulte et al. 2010; Tuni and Berger-Tal 2012). To date, evidence exhibits that females emit volatile, silk-bound and body-bound sex pheromones for mate attraction and that male discriminatory behaviour is based on such pheromones (Gaskett 2007; Thomas 2011). Pheromones provide males with information about the female and may change with female age, sexual maturity and mating status. Studies have shown that virgin females produce sex pheromones to attract males while the pheromone could not be found in mated females (Chinta et al. 2010; Jerhot et al. 2010). Until now the causes for the absence of pheromones in mated females remain

questionable (Thomas 2011). It could be a male strategy to avoid sperm competition and thus increase paternity by inhibiting female pheromone emission e.g. by transferring chemical substances (Arnqvist and Rowe 2005) or it could be a female strategy to control mate attraction (Thomas 2011). Mated females may benefit from stopping pheromone production if the costs associated with multiple mating outweigh the benefits (Stoltz et al. 2007). For example, in the Australian St. Andrew's cross spider *Argiope keyserlingi*, females experience a reduction in prey capture and an increase in predation risk by the presence of males in their webs (Herberstein et al. 2002). However, for some mated females it might be beneficial to re-advertise receptivity if the benefits to mate again exceed the costs of polyandry at that time. This could be found in the Australian redback spider *Latrodectus hasselti* in which females release sex pheromones three months after mating to refill their sperm storage and thus to maximise their reproductive output (Perampaladas et al. 2008). Furthermore, mated females may increase their fitness if they strategically control the production of pheromones depending on the quality of their first mating partner. By advertising receptivity after mating with an unsuitable male, females may increase the opportunity to mate again and reduce the risk of fertilisation with males who carry incompatible genes by cryptically biasing paternity towards a preferred sire.

Here, we investigate whether strategic release of female sex pheromones underlies post-copulatory mate choice promoted by multiple mating in the orb-web spider *Argiope bruennichi*. In previous studies, *Argiope* females were never observed to reject related males (Welke and Schneider 2009; Welke and Schneider 2010) although they are likely to receive only a single sperm load from one male during their short reproductive season (Schulte et al. 2010; Zimmer et al. 2012), which could be a sibling. From the female point of view, it might be adaptive to be initially indiscriminate if she can thereby secure a mating first and then cryptically bias paternity towards a superior male in successive matings as shown in the congener *Argiope lobata* (Welke and Schneider 2009). This suggests that females mated with a sibling have a particularly strong interest of attracting another male and thus



would benefit from continuing advertising via sex pheromones. Usually, *Argiope* males are not attracted to mated females as they will expect reduced reproductive success due to the presence of mating plugs (Nessler et al. 2007), which explains that many mated females often receive no second copulation in the field (Welke et al. 2012). However, females of orb-web spiders possess paired sperm storage organs (spermathecae) that connect to the outside via independent insemination ducts and genital openings (Eberhard 2004; Foelix 2011), so that the presence of a mating plug in one of them does not prevent copulation into the unused and unplugged genital opening. Thus, females that had mated once would have one genital opening unused and males that encounter a half-virgin female that has mated with a brother in her first copulation (sib half-virgin) can expect a higher paternity share than males that choose to mate with a half-sided virgin female that already copulated with a non-sibling male (non-sib half-virgin) due to post-copulatory female choice. Since double matings occur in the field (Welke et al. 2012; Zimmer et al. 2012), we expect that males should prefer sib half-virgins over non-sib half-virgin females if no alternative mating partners are available. As we know from previous studies that *Argiope* males use airborne and silk-based sex pheromones to distinguish between females of different quality (Herberstein et al. 2002; Gaskett et al. 2004; Schulte et al. 2010; Zimmer et al. 2014b), we used a silk-based choice experiment to test the hypothesis that mated females are more attractive if they had copulated with a sibling rather than if they had mated with a non-sibling male.

## Material and Methods

### Study species

The orb-web spider *Argiope bruennichi* occurs commonly in the Mediterranean area, but has expanded its range to Central and Northern Europe during the last century (Guttmann 1979; Kumschick et al. 2011). It inhabits dry meadows with patchy vegetation as well as marsh areas in which they can build their large orb-web a little above the ground (Bellmann 2006, 2010). The females are much bigger than the males and cannibalise up to 80% of their male mates after mating (Schneider et al.

2006). Female spermathecae can be filled by the same or different males in two separate copulation bouts (Uhl 2002; Nessler et al. 2007). Corresponding to females' paired genitals, males have two secondary copulatory organs (pedipalps) that they use to transfer their sperm into female spermathecae (Foelix 2011). Thereby, the complex morphology of male genital apparatus together with the external female morphology permits only a fixed ipsilateral insemination pattern (Uhl et al. 2007), i.e. males can only use the right pedipalp to insert into female's right genital opening and the left pedipalp into female's left genital opening.

### Study animals and laboratory maintenance

We collected egg sacs from a meadow in Nebenstedt, Northern Germany (53°09'69.48" N, 11°13'07.72" E), in April 2013. There were no specific permissions required for the sampling location. All hatchlings were raised to adulthood in the laboratory of the University of Hamburg. Each spider was individually labelled so that it was known from which egg sac it derived and could hence be unambiguously labelled as sibling.

Males were housed in individual upturned plastic cups of 250 ml. Females were initially kept in individual upturned plastic cups of 330 ml and transferred in individual Perspex frames (36 × 36 × 6 cm) after they moulted to maturity. Mate-choice experiments were conducted in these frames. All spiders were watered five days a week and fed twice a week. Males were fed with approx. 15 *Drosophila* spec. flies and females received three *Calliphora* flies. All spiders were checked daily for moults and were weighed on their day of maturation and on the day of experimentation on an electronic balance (Mettler Toledo AB54-S) to the nearest 0.001 mg. Males were weighed before trials, whereas females were weighed afterwards to prevent damage to the webs prior to experimental trials. The mean weight of males was  $17.4 \pm 0.7$  mg (N=30) and the mean weight of females was  $420.55 \pm 10.44$  mg (N=60) at the day of experimentation. All females and males that were used in the mate-choice experiments were frozen at - 80°C in the freezer in the laboratory for further measures. We measured the tibia-patella length of the first pair of legs as an indicator of body size under a dissecting microscope using the measuring tool of Leica

IM500. The mean leg length of males was  $4.86 \pm 0.1$  mg ( $N=26$ ; 4 missing data) and the mean leg length of females was  $7.6 \pm 0.1$  mg ( $N=60$ ).

For the mate-choice experiment, females were mated once with a single male that was either a sibling (hatched from the same egg sac) or a non-sibling (hatched from a different egg sac from the same population). As a previous study has shown that *A. bruennichi* populations are genetically diverse, even when they recently settled a meadow (Zimmer et al. 2014a), we can safely assume that most of the spiderlings from the different egg sacs were produced by unrelated females.

### Mate choice

We conducted mate-choice experiments over a period of seven days. In the experiment, virgin males were offered a choice between females that had mated either with a sibling (sib half-virgin) or a non-sibling male (non-sib half-virgin).

All spiders were tested only once and males were never related to the pairs of females. Females were chosen randomly from those of appropriate mating history, however, the two females were approximately size matched. Mated females were not used until at least two days after copulation and all individuals had to be adult for at least two days before the choice experiments. Female webs were always destroyed on the evening before the experimental day, so that females were forced to build fresh webs overnight which could be used for the experiments. To prevent a consistent position of females with the same mating history, frames with females in their webs were placed randomly to either side of the choice apparatus in every trial. Males showed no apparatus side preferences during experimental trials (19 right and 11 left; likelihood ratio test:  $\chi^2=2.16$ ,  $P=0.14$ ).

For each trial, we placed a sib half-virgin female in a frame adjacent to a non-sib half-virgin female in a frame (approximately  $100^\circ$  angle to each other) and connected a silk-thread from each web to the tip of a 20 cm long wooden skewer. The wooden skewer was fixed at an angle of around  $30^\circ$  to the bench top between and in front of the two frames (Gaskett et al. 2004). We used a new wooden skewer for each trial. We started trials by gently placing the male with a paintbrush at

the bottom of the wooden skewer. As spiders tend to walk upwards, males reached the end of the skewer and touched the two silk threads. Usually, they paused for a while before making the choice and then walked along one of the threads to the corresponding web. Trials in which males entered a web without having touched both threads were discarded. We recorded the time from placing the male on the skewer until touching both silk threads and the time from touching both threads until making a decision for one of the two females, as well as the time from making a decision until entering female's web. As soon as the male entered the web of the chosen female, we observed male and female behaviour until copulation or until the male spent one hour in the web. We documented whether copulation occurred or not, the copulation duration and whether males were cannibalised or escaped female's attack.

### Statistics

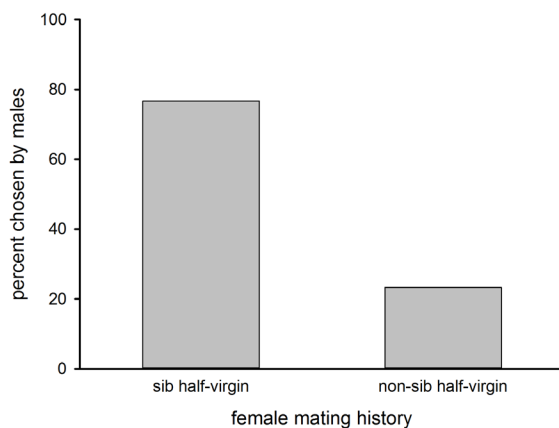
Data analyses were conducted using the statistical program JMP 7.0.2. Data were checked for normal distribution and equal variances. Data were transformed if the requirements for parametric tests were not met. Most data were analysed using likelihood ratio tests that are indicated in the results. Otherwise, we used an ANOVA and a linear regression as implemented in JMP. A logistic model was used to analyse whether mate choice depended on female age, size and weight. A pairwise correlation was performed to analyse data from the size-assortative choice experiment. All summary statistics are reported as mean  $\pm$  standard error (SE).

### Results

In total, 30 males fulfilled the requirements of a successful trial (touching the threads of both webs) and performed a mate choice in which they followed one of the threads and entered the web of the chosen female. It took males on average  $88.73 \pm 28.35$  sec. until they made a decision. However, as soon as they had made a choice they only needed  $4.67 \pm 0.53$  sec. to enter the web of the chosen female.

Males showed a significant preference for females that had previously mated with a sibling (likelihood ratio test:  $\chi^2_1=8.99$ ,

$P=0.0027$ ,  $N=30$ ; 76.7% of males chose sib half-virgin females; Figure 1). Overall, male choice was independent of the age of females ( $\chi^2_1=0.48$ ,  $P=0.49$ ) and female size ( $\chi^2_1=0.54$ ,  $P=0.47$ ) or weight ( $\chi^2_1=0.08$ ,  $P=0.79$ ; logistic model:  $\chi^2_3=2.12$ ,  $P=0.55$ ,  $N=30$ ). We found no evidence for size-assortative choice, as neither were male and female body size correlated ( $r=0.17$ ,  $P=0.4$ ;  $N=26$ , 4 missing data) nor the body weights of the males and the females ( $r=0.13$ ,  $P=0.49$ ,  $N=30$ ).



**Figure 1.** Summarised results of the mate-choice experiments including the percentage of virgin males that chose either females that had mated with a non-sibling male ( $N=7$ ) or females that had mated with a sibling male ( $N=23$ ).

Male mate choice was followed by copulation in 27 out of 30 males (90%) with the chosen female within one hour. The remaining 3 pairs were not different in female mating history (likelihood ratio test:  $\chi^2_1=0.17$ ,  $P=0.68$ ,  $N=30$ ). On average, males copulated for  $11.05 \pm 6.07$  sec. with non-sib half-virgin females and  $7.97 \pm 1.06$  sec. with sib half-virgin females. Female mating history had no effect on copulation duration (ANOVA:  $F_{1,25}=0.01$ ,  $P=0.92$ ). The copulation duration was independent of the weights of the chosen females (linear regression:  $F_{1,25}=2.49$ ,  $P=0.13$ ). Cannibalism occurred in 20 of 27 cases (74.07%) after mating independently of female mating history ( $\chi^2_1=0.37$ ,  $P=0.54$ ,  $N=27$ ).

## Discussion

In this study, we investigated whether mate attraction depends on female's need for additional matings to trade-up for superior males. Our results revealed that half-virgin

females that had mated with a sibling were more attractive for virgin males than half-virgin females that had received a mating with an unrelated male. Furthermore, we demonstrated that mating behaviour was not influenced by female mating history.

Our results support the strategic pheromone production hypothesis as females seemed to be able to adapt the release of sex pheromones to their benefits of attracting another, ideally superior mate. This suggests that the benefits of multiple mating only exceed the costs that are associated with mate attraction and pheromone synthesis if the first male was suboptimal. In *Argiope bruennichi*, a single sperm load is sufficient to fertilise all eggs (Schneider et al. 2005). Our results suggest that multiple mating per se is not beneficial for females. In contrast, polyandry requires an additional investment by females in those systems in which females attract males (Umbers et al. unpublished) and particularly in which the even sex ratios and sexual cannibalism constrain mating rates of both sexes.

Pheromone production is associated with costs (Johansson and Jones 2007; Foster and Johnson 2011; Harari et al. 2011). In the redback spider *Latrodectus hasselti* females recommence pheromone production after producing multiple egg sacs, probably to compensate depleted sperm supplies (Perampaladas et al. 2008). In our study, the main benefit of multiple mating is obviously genetic compatibility. Females that continue advertisement after having mated with a related male increase the possibility of receiving another mating and to thereby cryptically choose. Females may bias paternity towards a compatible male or they may simply increase the genetic diversity of their offspring (Simmons 2005; Cornell and Tregenza 2007).

The option of post-copulatorily biasing paternity in favour of one ejaculate over another is particularly likely in entelegyne spiders that possess a complex genital morphology with two independent spermathecae (Uhl 2002; Eberhard 2004). Indeed, cryptic female choice has been shown in our study species (Schneider and Lesmono 2009) as well as in the congener *Argiope lobata* (Welke and Schneider 2009). Females of *A. lobata* are able to vary in the number of stored

sperm in their spermathecae and reduce the storage of sperm from related males and thus the paternity of siblings towards genetically dissimilar males leading to the avoidance of genetic incompatibility (Zeh and Zeh 1997; Tregenza and Wedell 2000; Simmons 2005) or inbreeding depression (Pusey and Wolf 1996; Keller and Waller 2002).

Until now, there is little information about the production and the control of emission of sex pheromones in female spiders. It is known that females use silk or webs for pheromone transmission and that contact pheromones from silk may provide more specific information about female's identity and mate quality than silk or cuticular pheromones received via airborne cues (Gaskett 2007). Furthermore, female sex pheromones vary in the composition and ratio of chemical components among individual females (Symonds and Elgar 2008) which may cause the different responses of males to those chemical signals.

In the wolf spider *Schizocosa malitiosa*, females up-regulate the concentration of sex pheromones when they remain unmated for too long. High concentrations are emitted by old virgin females compared to mated or recently mated females (Baruffaldi et al. 2010). The signalling function of the different pheromone concentrations were confirmed by silk-based mate choice experiments showing that *S. malitiosa* males prefer old virgins over young virgins or mated females (Baruffaldi and Costa 2014). In other spider species, females not only vary the concentration but also the chemical composition of pheromones (Trabalon et al. 1996). Mostly, females produce sex pheromones as virgins and then cease the production after mating such as in the desert spider *Agelenopsis aperta* (Papke et al. 2001) and in the redback spider *Latrodectus hasselti* (Stoltz et al. 2007). The strategic release of pheromones by females could also be demonstrated in insect species, e.g. in the moth *Lobesia botrana*, in which a change in the amount of pheromone components underlies male discrimination among females of different reproductive value (Harari et al. 2011). According to a chemical study (Chinta et al. 2010), we assume that *A. bruennichi* females stop producing pheromones or vary their concentration rather than the chemical composition if they no longer benefit from further mate attraction. However, further

studies are required to verify this assumption as the pheromone blend contains at least one other specific compound of unknown function (Chinta et al. 2010). We cannot entirely exclude the possibility that male preferences are not entirely induced by female signalling activity. Males have less to gain from mated females and benefit from the ability to assess female mating status. They may evolve an ability to use cues that are not intentionally displayed by females. Nevertheless, our results suggest that at last an interaction between female signalling and male interest is responsible for the enhanced attractiveness of sib half-virgin females.

Whether a female had mated with a sibling or a non-sibling male had no influence on the mating behaviour of successive matings. One explanation might be that virgin males generally aspire to survive the first copulation as they have then the opportunity to re-mate with another female. A previous study has shown that on the one hand a longer copulation facilitates males to transfer more sperm during copulation (Schneider et al. 2006) resulting in an increased paternity success. However, a long copulation is also attended by an increased risk of cannibalism as the potential of cannibalism during first mating is a direct function of copulation duration (Fromhage et al. 2003; Welke and Schneider 2012). *A. bruennichi* males that copulated longer than 10 sec. most likely get cannibalised by the female while males that jump off before 10 sec. have a higher chance to survive the female's attack (Schneider et al. 2006). Despite the absence of differences in mating behaviour, males that copulated with a female that had received a mating with a sibling can expect a higher paternity share towards their previous rival due to cryptic female choice.

In conclusion, our study showed that mated females adapt mate attraction on the benefits of polyandry. Females that had mated with a sibling seem to strategically continue advertising receptivity and thus attract further males. The benefit is that the presence of multiple ejaculates enables them to cryptically bias paternity towards a more genetically compatible mate. Instead, mated females that had mated with an unrelated male seem to stop producing sex pheromones possibly to minimise the costs associated with mate attraction. However, further studies are

absolutely essential to determine pheromone chemistry and intensity of the sex pheromones produced by female spiders to better understand the behavioural function of pheromones. This is to our knowledge one of the few experimental studies that investigated the potential that pheromone production might be related to the benefits of polyandry.

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## Chapter 4

### Opportunistic mate-choice strategies in the orb-web spider *Argiope lobata*

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Mate choice is an important evolutionary process that is often studied using binary choice tests, where an individual is presented simultaneously with a pair of mates of different quality. However, in many species sequential mate choice is common under natural conditions, which excludes direct comparisons of potential mates. Hence, being choosy includes an element of uncertainty of finding a better or another potential mate at all and may lead to a risk of remaining unmated. In this case, it has been argued that individuals should mate with the first mate they encounter to ensure fertilisation success, but subsequently mate preferentially with mates of higher genetic quality (trade-up hypothesis). In this study, we tested this hypothesis by investigating sequential mate choice in the spider *Argiope lobata*. We offered females two males that were either related or not in various combinations. We further intensified the negative effects of inbreeding, expecting that this would increase the benefits of choosiness, and used individuals with and without a history of inbreeding. As *A. lobata* males follow a monogynous or a bigynous mating strategy and the polyandrous females rarely mate more than twice, both sexes are under selection to choose a mating partner of the highest possible quality. Consistent with the trade-up hypothesis, we found that virgin mating pairs were unselective towards a mate, but once-mated spiders were extremely reluctant to mate with an incompatible mating partner. However, we found no significant influence of inbreeding history on mating decisions. Our findings suggest that our study spiders use a simple rule of thumb of mating randomly as virgins but reject incompatible mates when mated.

**Keywords:** spider, Araneidae, polyandry, trade-up mate choice, sequential mating, mating strategy

#### Introduction

Benefits of mate choice have been identified in many studies covering most taxa (Mays and Hill 2004; Neff and Pitcher 2005; Kempenaers 2007). However, the non-random selection of mating partners is also associated with costs, such as energy expenditure (Watson et al. 1998; Vitousek et al. 2007) and lost opportunities (Real 1990; Bonduriansky 2001). Furthermore, direct comparisons of mate quality may be challenging as many species rarely encounter potential mates simultaneously in nature (Gibson and Langen 1996; Jennions and Petrie 1997; Barry and Kokko 2010). Choosiness is particularly problematic when there is uncertainty about finding another mate of higher quality or another potential mate at all. Hence, in the worst case, rejection of a potential first mate may lead to complete reproductive failure (De Jong and Sabelis 1991). The trade-off between the benefits of choosing high-quality mates and

the costs of remaining unmated should have a strong influence on individual mating decisions. A long known solution to this problem is that individuals mate with the first potential mate they encounter to secure fertilisation success, but then try to mate with mating partners of higher genetic quality (Halliday 1983; Jennions and Petrie 2000). Thus, they should be unselective first and choosy in subsequent matings. Such a trade-up mate choice has been observed in a wide range of taxa, such as crickets (Bateman et al. 2001), guppies (Pitcher et al. 2003), newts (Gabor and Halliday 1997), and lizards (Laloi et al. 2011).

Most studies on mate choice have focused on the female, as it is the sex with the larger parental investment in offspring in many mating systems. If parental investment is asymmetrical, the sex with the larger investment benefits more from choice (Bateman 1948; Andersson 1994). However, males are also expected to show mate preferences under certain conditions (Bateman and Fleming 2006). This applies if male mating

opportunities are limited by high reproductive costs including mating effort and if there is variation in female quality (Bonduriansky 2001; Edward and Chapman 2011). The above mentioned conditions can be found in spiders, particularly in those species in which males invest maximally in mating with a single or maximally two females. Monogynous mating systems evolved several times independently in spiders and males may invest terminally into a single or a conditional strategy in maximally two females (bigyny) (Schneider and Fromhage 2010). Here, both sexes are under selection to choose a mating partner of the highest possible quality. As most spiders have a non-resourced mating system (males provide no resources and no parental investment), female choice will be based on male genetic quality and increase the fitness of offspring due to good genes and/or genetic compatibility of the mating pairs. Male choice will be based on female fecundity, which equals size and weight in invertebrates and on genetic quality as well. Note that costs of incompatibility are at least as high for males as for females under monogynous conditions. One subject of genetic incompatibility is the degree of relatedness or genetic similarity between mates (Tregenza and Wedell 2000). Inbreeding generally results in reduced offspring fitness through the increase of homozygous deleterious alleles or the loss of heterozygosity (Charlesworth and Charlesworth 1987; Charlesworth and Willis 2009) and mechanisms to avoid or reduce matings between relatives are well documented (Pusey and Wolf 1996; Keller and Waller 2002). In spiders, it has been proposed that female multiple mating has evolved to facilitate post-copulatory choice of a preferred male and experimental studies demonstrated post-copulatory selection against sperm of siblings (Welke and Schneider 2009), and against sperm of males that had courted less (Stoltz et al. 2008; Schneider and Lesmono 2009).

The strength of selection on choosiness will differ depending on environmental conditions and expected gains (Jennions and Petrie 1997; Bleu et al. 2012). In scenarios in which low mobility (Levitan 2004), low population density (Moller 2003; Kokko and Rankin 2006), a biased operational sex ratio (Jiggins et al. 2000; Schmidt et al. 2014), a temporary reproductive season (Friberg and Wiklund 2007) or a short live span (Wickman 1992) constitute a risk of

remaining unmated, individuals should be less choosy. In contrast, individuals that are confronted with a high risk of inbreeding are expected to become increasingly choosy, particularly if they themselves derived from an inbred mating. In many spiders, demographic conditions are changing quickly during a short reproductive season and females are sedentary and encounter males sequentially in nature (Kasumovic et al. 2008; Zimmer et al. 2012). Thus, individuals should balance the probability of remaining unmated against the fitness losses through incompatible mating partners.

In this study, we experimentally varied the fitness consequences of random mating in two ways; firstly by increasing the probability of homozygosity through different histories of inbreeding and secondly by providing a sibling or a non-sibling as a first mating partner. Many species show some tolerance to inbreeding such that negative effects will only be apparent after two generations of inbreeding (Bilde et al. 2007) so that the need to reduce the negative effects of inbreeding is larger if homozygosity is already high. We predict that spiders in the second generation of inbreeding suffer higher costs when mating with a sibling and should therefore be particularly receptive towards a second more compatible partner, particularly if they have mated with a sibling first. Conversely, these individuals should reject another incompatible male. To test these predictions, we used the sexually cannibalistic spider *Argiope lobata* that does not show pre-copulatory discrimination against siblings when virgin, although polyandrous, outbred females are known to reduce costs of inbreeding through post-copulatory cryptic choice (Welke and Schneider 2009). We mated offspring derived from wild-caught egg-sacs to create an inbred or outbred  $F_1$  generation. In sequential choice tests, inbred and outbred females from the  $F_1$  generation were offered two male spiders of different relatedness in five treatment combinations (see Table 1): (1) two copulations with the same non-sibling male, (2) two copulations with the same sibling male, (3) two copulations with different non-sibling males, (4) first a sibling and then a non-sibling, or (5) first a non-sibling and then a sibling. We recorded female and male mating behaviour and the latency until mating occurred.

According to the trade-up hypothesis, we predicted that females and males would be more willing to accept a second mate if the second mating partner is genetically more compatible than the previous partner. The benefits of this strategy should be intensified with increasing inbreeding. This leads to the following predictions for the five different treatments (see Table 1): (1) they should mate twice with the same non-sibling in part of the cases, (2) they should refuse to mate with the same sibling during second mating, (3) they should generally accept to mate with a different non-sibling during second mating, (4) they should readily mate with a non-sibling after mating with a sibling, (5) they should be reluctant towards a sibling during second mating after mating with a non-sibling. As we cannot precisely determine female and male contributions to the mate choice under our no-choice design, we assumed mutual mate choice in this study.

## Material and Methods

### Study Animal

For this study, we used the orb-web spider *Argiope lobata* which can be found in Africa, Europe and Asia (Platnick 2013). It prefers sunny and dry places where it builds its typical orb-web with the characteristic stabilimentum in the middle (Bellmann 2010). Sexual dimorphism is pronounced in that males are small and inconspicuously coloured in comparison to the females, which have a silvery coloration and an opisthosoma extended by lobes. All spider males have two secondary mating organs, the pedipalps, and all entelegyne spider females have two genital openings that end in two separated sperm storage organs, the spermathecae, which can be filled from different males (Foelix 2011). *Argiope* males have so called one-shot genitalia that are used to plug female genital openings (Nessler et al. 2007). The plugging induces damage to the male mating organ (pedipalp) and renders the pedipalp useless (Fromhage and Schneider 2006).

All spiders used for the mating experiment in this study were  $F_1$  descendants derived from matings between siblings and non-siblings that had hatched from field-collected egg-sacs (near Ashkelon, Israel). The collected egg-sacs

were kept in the laboratory of the University of Hamburg. Spiderlings that hatched from those egg-sacs (generation called P for parental) were raised in the laboratory until they reached adulthood. Each spider was individually labelled so that it was known from which egg-sac it derived. Spiders from the same egg-sac were considered to be siblings, but since the mother may have mated more than once, they might have been half-siblings too. They were kept in individual plastic cups (depending on body size in 250 ml or 330 ml cups) and sprayed with water on five days of the week. Depending on their developmental stage and body size they were either fed ad libitum with *Drosophila spec.* or with three *Calliphora* flies twice a week. Once the females reached adulthood they were transferred in individual Perspex frames (36 · 36 · 6 cm), where they built their typical orb webs. We randomly selected males and females to either mate as pairs of siblings and as non-siblings. One male and one female were put together inside the same frame for two days. Thereby, we secured at least one copulation. Subsequently, mated females were transferred from the frames into 500 ml plastic cups where they built their egg-sacs. Spiderlings that hatched from those egg-sacs ( $F_1$ ) were raised under the same conditions as mentioned above and used for the mating experiment as soon as they moulted to maturity. Hence, females and males from the  $F_1$  generation either derived from sibling matings (inbred) or from non-sibling matings (outbred).

### Experimental Design

Mating experiments were conducted from February until May 2011. Female virgins were mated first with a virgin male (a single copulation was permitted) and a second time with a once-mated male. Males were weighed prior to every mating trial while females were weighed afterwards to prevent damage of their webs. We used 57 inbred and 14 outbred females and assigned them to five different double mating treatments in which they were introduced (1) to the same sibling twice (S2x), (2) to the same non-sibling twice (N2x), (3) to two different non-siblings (N/N), or (4 & 5) to one sibling and one non-sibling male in random order (SN and NS).

Males use only one pedipalp per copulation and always insert their right pedipalp in the

right genital opening and the left pedipalp into the left genital opening (Uhl et al. 2007). This fixed ipsilateral insertion pattern enables us to form mating pairs for the second mating in which a once-mated male inseminates an unused spermatheca of a once-mated female with his unused pedipalp.

A mating trial began when a male was gently placed in the corner of a female's web (inside the Perspex frames). Normally, males start to vibrate in the web while carefully walking to the female in the hub. Once they reach the female, they start courtship by carefully stroking the female's legs. Receptive females assume a copulation position by putting their opisthosoma in a horizontal position. This enables the male to sit on the opisthosoma and insert his pedipalp in one of the two genital openings. 30-40% of *A. lobata* males are cannibalised by the female after this first copulation (Nessler et al. 2009). We prevented sexual cannibalism during the first copulation by gently holding a paintbrush between the female's chelicerae and male's body. All males died during their second copulation as is normal for the genus and were removed from the web before females could eat them. Males were kept and frozen at -80 °C for further measurements. During every mating trial, we noted male and female mating behaviour, the time from first web contact until mating, the beginning and duration of copulation, the insemination duct the male copulated into, as well as the cannibalism rate (only at second mating). Furthermore, we recorded the number of trials until first and second mating occurred, the number of replaced males until the final male mated and whether two matings were successful or not. Virgin pairs always mated with each other during first mating, but not always within the first trial. If a mating did not occur within the first trial (each trial lasted 120 minutes), we introduced the same male to the same female again after a short break on the same day and/or on the following day. However, if mating did not happen within three trials (which occurred only in 8 of 71 cases (11.27%) during first mating), we replaced the male with a new male and introduced the new male to the same female until mating occurred or for a maximum of another three trials. Immediately after the first copulation the second mating was induced by placing a mated male into the

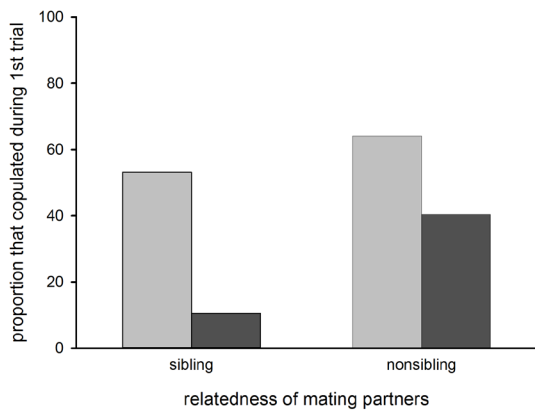
web with the female. Most once-mated pairs were reluctant to mate. If a mating did not occur within the first trial, we introduced the same male to the same female again after a short break on the same day and/or on the following day. However, if mating did not happen within the first three trials, we replaced the male with a new male and followed the trial scheme mentioned above every day until mating occurred or the female produced an egg-sac. Only in the treatment S2x and N2x in which females had to mate with the same sibling or non-sibling twice, males were not replaced with a new male during second mating trials and were introduced to the same female until the second copulation occurred or the female produced an egg-sac.

### Statistics

Data analyses were carried out with JMP 7.0.2 and most data were analysed using likelihood ratio tests. Continuous data were inspected for normal distribution and equal variances. Data were transformed (log) if the requirements for parametric tests were not met and analysed with a parametric t-test. If transformations were not possible, the non-normally distributed data were analysed with the non-parametric Wilcoxon test. The influence of several factors on the success of matings during first trials was analysed with a logistic model. All tests are indicated with the results. Descriptive statistics are given as mean  $\pm$  standard error (SE) and percentages (%).

### Results

In total, we mated 71 pairs once, of which 42 pairs (59.15%) showed no reluctance and mated during the first mating trial. The remaining 29 pairs (40.85%) mated after  $3.9 \pm 0.77$  trials. Whether or not the first mating occurred during the first trial was independent of the relatedness of the mating partners ( $\chi^2_1=0.98$ ,  $N=71$ ,  $P=0.32$ ; see Figure 1), the breeding history of the female ( $\chi^2_1=0.17$ ,  $N=71$ ,  $P=0.68$ ), the male ( $\chi^2_1=3.01$ ,  $N=71$ ,  $P=0.08$ ), and the interaction between the relatedness of the mating pairs and female breeding history ( $\chi^2_1=2.3$ ,  $N=71$ ,  $P=0.13$ ; logistic model:  $\chi^2_4=3.93$ ,  $N=71$ ,  $P=0.42$ ).



**Figure 1.** Percentage of copulation that occurred during 1<sup>st</sup> trials in double-mating experiments (grey = 1<sup>st</sup> copulation; dark = 2<sup>nd</sup> copulation).

However, mating pairs were less willing to mate during their second mate encounter, resulting in only 23 pairs (32.4%) that re-mated during the first mating trial and 30 pairs (42.25%) that mated after  $5.47 \pm 0.79$  trials. Eighteen of 71 pairs (25.35%) never mated a second time despite  $8.83 \pm 2.03$  trials over a period of  $6.22 \pm 1.34$  days. These pairs were distributed across all but the N2x treatment (0% N2x; 27.78% S2x; 33.33% N/N; 5.56% N/S; 33.33% S/N; see Table 1).

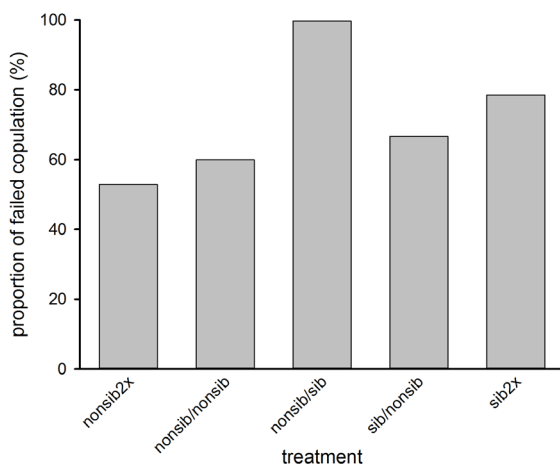
The relatedness of the second male to the female was the only variable that explained whether or not a second mating occurred during the first trial ( $\chi^2_1=7.05$ ,  $N=71$ ,  $P=0.008$ ; see further results below). The breeding history of the female ( $\chi^2_1=0.23$ ,  $N=71$ ,  $P=0.63$ ), the male ( $\chi^2_1=0.36$ ,  $N=71$ ,  $P=0.55$ ), and the treatment (first male related or not:  $\chi^2_1=1.15$ ,  $N=71$ ,  $P=0.28$ ; interaction first male and second male:  $\chi^2_1=1.39$ ,  $N=71$ ,  $P=0.16$ ; see Figure 2) were not significant. Furthermore, the interaction between the relatedness of the second male and female breeding history ( $\chi^2_1=0.24$ ,  $N=71$ ,  $P=0.62$ ; whole logistic model:  $\chi^2_6=9.79$ ,  $N=71$ ,  $P=0.13$ ) had no influence on the mating success during the first trial. In addition, the occurrence of second matings during the first trial was independent from whether the second mating partner was the same male or a different one (likelihood ratio test:  $\chi^2_1=0.24$ ,  $N=71$ ,  $P=0.63$ ).

In 19 different trials a sibling was introduced to the web of a once-mated sister and copulation occurred in only two cases (10.53%) during the first trial (see Figure 1). In 11 cases (57.89%) mating occurred only after a mean of  $6.45 \pm 1.63$  repeated introductions. In the remaining six trials (31.58%) a mating never occurred. Repetitions of mating trials were terminated when the female produced an egg-sac, which occurred after a mean of  $18.17 \pm 3.89$  repetitions.

**Table 1.** Summary of the treatments, predictions and corresponding findings for the five different sequential mate-choice trials.

Treatment	Prediction	Finding
(1) two copulations with the same non-sibling male	(1) pairs should mate twice in most cases	(1) all pairs re-mated (47.06% in 1 <sup>st</sup> trials, 52.94% after multiple trials)
(2) two copulations with the same sibling male	(2) pairs should refuse to mate with the same sibling during 2 <sup>nd</sup> mating	(2) 35.71% refused to mate with the same sibling twice; 64.29% re-mated (33.33% in 1 <sup>st</sup> trials, 66.67% after multiple trials)
(3) two copulations with different non-sibling males	(3) they should generally re-mate	(3) 60% re-mated (66.67% in 1 <sup>st</sup> trials, 33.33% after multiple trials); 40% did not mate after repeated trials
(4) first a sibling and then a non-sibling	(4) they should readily re-mate	(4) 66.67% re-mated (50% in 1 <sup>st</sup> trials, 50% after multiple trials); 33.33% did not mate after repeated trials
(5) first a non-sibling and then a sibling	(5) they should not re-mate	(5) 14.29% did not re-mate after repeated trials; 85.71% re-mate, but were extremely reluctant (all after multiple trials)

Copulation was more likely to occur when an unrelated male was introduced to a once-mated female: in 21 of 52 cases (40.38%) a second copulation took place during the first trial (see Figure 1). However, even non-siblings required a mean number of  $4.89 \pm 0.83$  trials in 19 cases (36.54%) and 12 trials (23.08%) were unsuccessful. Re-mating among non-sibling partners was more likely to occur between the same mating partners (in all 17 cases: 100%) than with novel mating partners (in 9 of 15 cases: 60%).



**Figure 2.** Percentage of pairs that failed to copulate in each of the five treatments during the first trial of the 2<sup>nd</sup> mating.

We measured copulation duration and cannibalism rate in all successful matings. The mean duration of copulation was  $24.87 \pm 3.09$  sec. for the first mating ( $N=67$ , 1 outlier was excluded, 3 missing data points) and  $87.12 \pm 18.06$  sec. for the second mating ( $N=47$ , 1 outlier was excluded, 5 missing data points). Copulation durations of siblings and non-siblings did neither differ in first (Wilcoxon test:  $Z=-0.25$ ,  $N=67$ ,  $P=0.51$ ; 1 outlier was excluded; 3 missing data points) nor in second matings (t-test:  $t=-0.35$ ,  $N=47$ ,  $P=0.72$ ; 1 outlier was excluded; 5 data points missing). Furthermore, copulation duration was independent of whether females and males of mating pairs derived from inbred or outbred matings during first matings and during second matings (Table 2). Sexual cannibalism occurred in 52.83% of second matings (28 of 53) and did not differ between siblings and non-siblings (likelihood ratio test:  $\chi^2_1=0.53$ ,  $N=53$ ,  $P=0.47$ ). Moreover, male and female breeding history had no influence on the occurrence of sexual cannibalism during second matings (Table 2). Note that sexual cannibalism was prevented during first mating.

**Table 2.** Summarised results of the dependency of female and male breeding history on the copulation duration and cannibalism rate during double-mating experiments.

	Females				Males			
	Inbred	Outbred	Test	P	Inbred	Outbred	Test	P
1 <sup>st</sup> Copulation duration (sec.)	$26.2 \pm 3.6$ (54)	$19.6 \pm 5.5$ (13)	$Z = -0.86$	0.38	$26.1 \pm 5.4$ (34)	$23.6 \pm 3.1$ (33)	$Z = 0.87$	0.38
2 <sup>nd</sup> Copulation duration (sec.)	$94 \pm 22$ (34)	$69.2 \pm 31.8$ (13)	$t = 0.78$	0.45	$56.6 \pm 14.3$ (21)	$111.8 \pm 30$ (26)	$t = 0.29$	0.77
Cannibalism	46.15 % (18 of 39)	50 % (7 of 14)	$\chi^2_1 = 0.06$	0.81	45.6 % (10 of 22)	48.39 % (15 of 31)	$\chi^2_1 = 0.04$	0.83

## Discussion

In sequential mating trials, we found evidence supporting the trade-up hypothesis in the sexually cannibalistic spider *Argiope lobata*. As predicted, all unmated spiders readily mated with the male that was provided (although 40% of spiders required more than one trial), independent of the genetic match of mates.

Females and males did not display discrimination at their first mating opportunity. During second mating opportunities, mating pairs were highly reluctant to mate with each other when their mating partner was a genetically incompatible mate. These results suggest that unmated *A. lobata* will mate with any mating partner, presumably to secure a baseline reproductive success, which is

fertilisation of the eggs for the female and paternity for the male. Subsequently, females and males will avoid copulations with incompatible mating partners. Acceptance of a second mate was neither influenced by the relatedness of the first mate, nor by the inbreeding history. Hence, *A. lobata* spiders seem to follow a simple rule of indiscriminate mating when virgin and avoidance of inbreeding when mated. Thereby, multiple mating will benefit both sexes on average but the spiders do not seem to use multiple mating as individual strategy of adjusting partner quality.

Consistent with the trade-up hypothesis, unmated spiders required fewer trials until mating occurred than once-mated spiders. The observed pattern is adaptive under conditions of high environmental uncertainty about mate availability, leading to a disadvantage by generally rejecting relatives as mating partners. Female web-building spiders are sedentary and do not actively search for a mate (Foelix 2011; Zimmer et al. 2012) and hence may indeed face a risk of remaining unmated. Likewise, *A. lobata* males may not reach a female, let alone a second one during their search, which is particularly so if population density is low. Limited male mating opportunities due to risky mate search and/or low population density can also be found in other species such as Australian redback spiders (Andrade 1996, 2003) or wolf spiders (Marshall et al. 2002), crickets (Kindvall et al. 1998) and other insect species (Fauvergue 2013). Low mate availability may stay at the same level throughout the mating season (Kokko and Jennions 2008) or may fluctuate depending on the time of the season (Kasumovic et al. 2008) which is the case in the congener *Argiope bruennichi* (Schulte et al. 2010; Zimmer et al. 2012). We have no quantitative information about such dynamics for *A. lobata* but assume the general pattern of protandry and a patchy distribution of females in the genus.

The observed more selective responses towards mating partners in subsequent mating opportunities partly confirmed the second prediction of the trade-up hypothesis namely that mated individuals should only mate with a mate of higher quality than the first (see below). This trade-up strategy has been reported in various taxa (Gabor and Halliday

1997; Bateman et al. 2001; Pitcher et al. 2003; Laloi et al. 2011) and most of these trade-up choices are based on secondary sexual traits associated with quality, but only few concentrated on cases in which individuals traded up for compatible genes (Laloi et al. 2011). Trading-up for compatibility has been reported in birds in which females frequently copulate with extra-pair males to obtain compatible genes and thereby increase the heterozygosity of their offspring (Johnsen et al. 2000; Foerster et al. 2003). Heterozygosity at the individual level of inbreeding is relevant for offspring survival as it reduces the expression of deleterious alleles. The trading-up strategy is often used to explain female multiple mating facilitating cryptic female choice (Jennions and Petrie 2000). Post-copulatory choice by females has been demonstrated in several *Argiope* species (Elgar et al. 2000; Schneider and Lesmono 2009) including *A. lobata* that use cryptic choice to favour sperm from unrelated males (Welke and Schneider 2009). Males of the congener *A. bruennichi* use a trade-up strategy as well. One-shot genitalia limit males to a maximum of two copulations, which they can accomplish with one or two females (monogynous or biygynous strategy). Males opt for a bigynous strategy if they first mate with a sibling or a small female (Fromhage and Schneider 2012; Welke et al. 2012). In such cases they will copulate only briefly with the first female, which increases their survival and the probability to find a better female for their second and last copulation (Welke and Schneider 2010).

According to the trade-up hypothesis, mated individuals should respond to a potential mate that is of higher quality than the first mate (Halliday 1983). Our results however revealed no significant influence of the previous mating partner's compatibility on the behaviour during a second mating opportunity. Rather, our mated spiders seemed to apply a general rule of avoiding siblings as mating partners while accepting compatible males. Nonetheless, we can assume that *A. lobata* spiders following this strategy will mostly gain a mate of higher or similar quality in successive matings as mating pairs. Mated spiders were extremely reluctant towards relatives after they had mated with a same quality or higher quality mate. Hence, the rejection of successive matings may be a result of the

failure to improve the reproductive output compared to the previous mate.

Not all individuals followed the above mentioned adaptive strategy. Some pairs that had mated with a sibling first were relatively reluctant during their second mating. This seems inappropriate, as individuals should adapt their responsiveness to the benefits obtained from the increased quality of the second mating partner. One explanation might be that mated females, even when they are non-siblings, are unattractive for males. This corresponds with other studies showing male's general preference for virgin over mated females (Gaskett et al. 2004; Schulte et al. 2010; Tuni and Berger-Tal 2012; Schneider et al. unpublished). The rejection of mated females is highly adaptive for mated males as they suffer a high risk of choosing a female whose virgin genital opening is incompatible with his unused pedipalp due to the fixed insemination pattern in *Argiope* (Zimmer et al. 2014). Thus, males that had their first mating with a sibling and consequently have the strong need to obtain an additional mating with a genetically compatible female might have a higher chance to compensate the reduced fitness costs of their first mating with a subsequent virgin and non-sibling female.

Surprisingly, our results revealed that non-sibling pairs always re-mated with the same mating partners, while matings between non-sibling pairs and different mating partners only succeeded in 60% during second mating opportunities. This finding refutes the widespread assumption that multiple mating per se and the acquisition of sperm from several males is generally desired by females to elevate their individual fitness (Zeh 1997). Indeed, several fitness benefits to females have been demonstrated in various taxa (Zeh and Zeh 2003; Simmons 2005; Slatyer et al. 2012). *A. lobata* spiders seemed to readily accept re-mating with the same partner which means likely monopolisation of paternity by a single male for the female and fertilisation success of the eggs of one female only for the male. Hence, the spiders do not resist monogamy if the mating partner is an unrelated/compatible male suggesting that in the absence of sibling partners the benefits of polyandry do not exceed the costs.

The duration of copulation and the rate of cannibalism did not differ between sibling and non-sibling pairs regardless of their mating status. These findings are consistent with previous observations of *A. lobata* (Welke and Schneider 2009). However, it has to be considered that we prevented females from attacking the males during first matings in this study design, which might have had an influence on copulation duration. Both, sibling and non-sibling males may have copulated for longer without the interference of the female. This may have resulted in the transfer of more sperm during copulation (Schneider et al. 2000; Elgar et al. 2003). In the congener *A. bruennichi*, males shorten their copulations when mating with a sibling (see above) (Welke and Schneider 2010). Killing a sibling during his first copulation and preventing him from achieving his potential reproductive success will not only severely limit fitness of the male but would lead to indirect fitness loss of the female.

While the dynamics of copulation duration and sexual cannibalism during the first copulation remain to be studied in a more natural set-up, the absence of behavioural differences between sibling and non-sibling pairs at their second mating opportunity can be explained: males can copulate only twice, hence mated males have no further mating opportunities and seek to prolong their final copulation to secure maximal sperm transfer and fertilisation success. Thus, the increased risk of cannibalism due to the prolonged copulation is not a determining factor any longer. On the contrary, it is rather beneficial to accept sexual cannibalism in *A. lobata* as prolonged copulation significantly increases plugging success (Nessler et al. 2009). The association of sexual cannibalism and plugging success implies that females should attack a male and consequently increase his paternity success if he is a genetically compatible mate while they should refrain from cannibalism and consequently decrease his plugging possibility and his paternity share if he is a sibling. However, previous observations demonstrated that females indiscriminately attack every male during copulation (Nessler et al. 2009) and choose post-copulatorily by controlling sperm storage. Indeed, the number of stored sperm is independent of copulation duration (Welke and Schneider 2009).



In conclusion, our results show that *A. lobata* females mate multiply to enhance their reproductive success by diluting the contribution of sperm from a first incompatible male. Males share female interests since they have a maximal mating rate of two and benefit from avoiding the negative effects of inbreeding. Hence, once-mated females and once-mated males are equally reluctant to copulate with a sibling. Virgin females and virgin males in contrast mate indiscriminately. Our results parallel mating strategies of birds and lizards, but differ in that re-mating selectivity is not strategic, at least not in the situation we simulated in our experiment. Rather, our study spiders seem to apply a simple rule of thumb to discriminate against incompatible males if sperm has been secured regardless of the quality of the sperm. Ideally, field experiments should be accompanied by genetic paternity analyses to assess the occurrence and benefits of trade-up strategies under natural conditions.

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## Chapter 5

### Context- and state-dependent male mate choice in a sexually cannibalistic spider

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High male mating investment may favor selection on male mate choice particularly if females vary in quality. However, sequential mate encounters and the risk of remaining unmated may reduce potential benefits of choice drastically. Terminal investment strategies constitute a maximal mating effort and have evolved independently in the absence of paternal investment in several spider taxa including the genus *Argiope*. To test for male mate preferences in the above context, we used the sexually cannibalistic spider *Argiope bruennichi*. We varied male state (mating status and post-maturation age) and the competitive context and quantified male mate choice decisions between females of different states and developmental stages in binary choice tests. We found an overall adaptive preference for the virgin against the mated female regardless of male mating state. Furthermore, we demonstrated that older males paid more attention to female fecundity related traits than to mating status. In a second set of experiments we offered males a choice between a virgin and a subadult female and varied the competitive context which had no effect on male decisions. Curiously a preference for the virgin adult female was only apparent after exclusion of females that matured less than three days prior to the test. Repeated tests of males supported the hypothesis that males cannot distinguish between a freshly matured virgin female and a subadult female. Our results add to the growing body of studies demonstrating male mate choice and the flexible state-dependent adjustment of preferences.

**Keywords:** sexual selection, monogyny, mating system, mating strategy, spider, Araneae, Araneidae

#### Introduction

Mate choice is expected when the benefits of selectivity exceed the costs and in some species this may be the case for both sexes, while in the majority of species the net benefit of choice is higher for one sex (Andersson 1994). Kokko and Mappes (2005) identified the costs of breeding as the most powerful selection pressure behind the evolution of choice and asymmetries in such costs will determine which sex will be more selective. The costs of breeding comprise the traditional concept of paternal investment and the operational sex ratio (OSR), but a large mating effort can be an equally important selection pressure on choosiness (Kokko and Monaghan 2001; Kokko and Johnstone 2002; Fawcett and Johnstone 2003; Servedio and Lande 2006). This concept of breeding costs is more general than parental investment as it includes mating systems, in which males provide no parental care and females mate multiply and it allows for the evolution of male mate choice under a

broader range of circumstances. Indeed, recent literature propagates that male mate choice is of wider occurrence than previously recognized and that it can evolve in the absence of paternal investment (Bonduriansky 2009; Edward and Chapman 2011). Males may benefit from being choosy if females vary in quality and mating opportunities are limited due to either intrinsic or extrinsic constraints (Servedio 2007). However, benefits of choice diminish if mate assessment is sequential rather than simultaneous and if rejection of a mate entails the cost of remaining unmated (Barry and Kokko 2010). While male mate choice is a largely established selective force in systems in which males provide investment in the offspring, it is still ambiguous under which conditions a high mating effort in the absence of paternal care favors the evolution of male mate choice.

Male mating effort is extreme in mating systems, in which males are physically constrained to mating with a single or maximally two females without providing

paternal care (Schneider and Fromhage 2010). Examples occur in various taxa, such as fish, crustaceans and insects but are particularly common in spiders. In mono- or bigynous mating systems of spiders, males invest their life and damage their genitals in order to maximize their paternity share (Nessler et al. 2007; Uhl et al. 2007; Kuntner et al. 2009; Kuntner et al. 2012). Intuitively, male mate choice is expected if males invest terminally into fertilizing eggs of a single female as their entire reproductive success will be determined by the fecundity, fertility and genetic quality of this female (Fromhage and Schneider 2012). Modeling approaches suggest that male mate choice can evolve if it is based on fecundity related traits (Servedio and Lande 2006). However, these benefits of choice need to exceed the manifold costs imposed by lost opportunities and diminished reproductive success due to increased competition for preferred females (Bonduriansky 2001). Furthermore, choice requires investment of time and energy, and the capacity of sampling and processing information, which may not evolve easily in small brained animals (Kokko and Mappes 2013). The question then becomes how large the costs and risks of sequential choice are and how they affect net fitness.

Importantly, the cost-benefit ratio can vary for individuals within a given population such that choosiness may pay off for only a proportion of the males while the remaining males are better off by jumping on the first opportunity (Venner et al. 2010). Such frequency dependent dynamics lower the competition over preferred mates and can thereby stabilize the existence of male mate choice (Servedio and Lande 2006). If differences in cost-benefit ratios for males are extreme and predictable, selection may favor alternative mating tactics or behavioral plasticity (Brockmann 2001; Bel-Venner et al. 2008). The latter requires individuals to assess their net benefit and make a decision about whether to be choosy or not based on their own internal state and on the circumstances (Venner et al. 2010).

We investigate the nature of male preferences and how they depend on state and context in a sexually cannibalistic spider, in which males have a maximal mating rate of two. Many spider species are known for first male sperm priority for example due to mating

plugs (Uhl et al. 2009). Such a precedence pattern favors a preference for virgin females, a pattern established in experimental studies of several species (Gaskett et al. 2004; Stoltz et al. 2007; Schulte et al. 2010; Tuni and Berger-Tal 2012). However, male preferences for virgin females can occur because males actively reject mated females or because mated females are unreceptive and do no longer signal their presence (Thomas 2011).

Incorrectly choosing a non-receptive female will entail costs that can vary in magnitude. Costs of incorrect choices are losses of energy and opportunities but may include losing life in sexually cannibalistic species (Schneider, in press). In many sexually cannibalistic animals, females vary in their propensity to attack a male and the variance may be related to female hunger and to female mating status (Wilder et al. 2009). By approaching a mated female, spider males may risk to lose their life before they were able to mate (Johnson 2001; Rabaneda-Bueno et al. 2008). Hence, it is reasonable that the existence of sexual cannibalism further enhances selective advantages of male choosiness (Schneider, in press). Barry and Kokko (2010) argue that in such species, male mate choice is nevertheless unlikely to evolve because of the high costs of sampling and the low probability of encountering several potential mates. However, reproductive synchrony and high densities will ensure high encounter rates and favor choice (Kokko and Johnstone 2002).

A recent model predicts that mate choice by males can evolve in species with low mating rates if females vary in their quality and due to seasonal variation in mate availability (Fromhage and Schneider 2012). The model predicts that mated males should be choosier than virgin males because of their larger opportunity cost (mated males always die after mating) but that choosiness should decrease towards the end of the season. The model further predicts that males employ conditional strategies related to the fecundity of the female. The model was inspired by the reproductive biology of *Argiope bruennichi*, a species in which the majority of males will not survive their first copulation. Survival probability after the first copulation increases after very short copulations and these occur more often with small and incompatible

females (Fromhage et al. 2003; Welke and Schneider 2010; Welke and Schneider 2012).

Here, we test males of the sexually cannibalistic orb-web spider *A. bruennichi* using an established simultaneous choice paradigm in the laboratory. Note that binary choice paradigms can only test for existence of preferences but provide no information on whether and how these preferences will be applied in nature. In a first experiment, we varied two male state variables, namely age (days since maturity) and mating status. The latter can take only two values in *A. bruennichi*, virgin or once-mated, as males invariably die after their second copulation (Schneider et al. 2006). Theory predicts that mated males should have a stronger preference for virgin females than virgin males (Fromhage and Schneider 2012) because it is their final mating opportunity. Male age should have an effect on choosiness because males mature before females and females mature within a short time frame of three weeks (Zimmer et al. 2012). Hence, young males would expect conditions early in the season with strong competition over high quality females. In addition, young males still have time ahead to encounter mating partners of higher quality and may therefore benefit from rejecting low quality females. In contrast, old males would find themselves later in the season and in addition to a reduced life expectancy they cannot expect to encounter many alternative mating partners, especially if they should be of higher quality than the current mate.

In the first experiment, males were given a choice between a virgin and a once-mated female. Females have two spermathecae that are independently connected to the outside via insemination ducts. During copulation, males insert one of their two pedipalps (secondary mating organs of spiders) into one of these insemination ducts. Hence, after one mating, the female still has one virgin spermatheca. It is known that males that mate into an unused spermatheca can expect a paternity share that is above or below 50% depending on relative copulation duration (Schneider et al. 2006).

In a second experiment we varied the competitive context and provided males with a choice between two females one of which had been visited by another male before. This potential rival had left a silken dragline

behind. Males are assumed to perceive the presence of another male via the dragline (Schneider et al. 2011). In addition, we varied female state by providing a choice between an adult virgin female and a subadult female. Subadult females are of interest to males even though they are not in their reproductive state yet. In the field, males can be regularly observed to sit and wait near and even in the webs of subadult females for days (Welke et al. 2012; Zimmer et al. 2012). Males wait for these females to molt and then mate with the molting and therefore defenseless female (opportunistic mating). Negative results of this experiment could be interpreted in two ways so that we designed a third experiment that tested whether males are unable to distinguish between subadult and young virgin females or whether half of the males prefers virgins while the other half prefers subadult females and opt for opportunistic copulations. We tested males repeatedly to investigate if males show consistent differences in their preferences.

## Methods

Adult male and female *A. bruennichi* derived from field collected egg-sacs, were raised from egg in the laboratory and used in three different binary choice experiments. Siblings were never used in the same trial (spiders hatched from the same egg-sac were considered siblings). The general procedure was the same in all experiments: two Perspex frames (30 x 30 x 6 cm) each containing a female spider with her web were arranged on a table with an angle of 120°. A silk thread was detached from each web and attached to the tip of a halved bamboo stick. The stick was fixed providing an upwards facing base for the males to walk on (see Gaskett et al. 2004 for schematic drawing of the set-up). The skewer and all instruments were washed with ethanol after each trial. A trial was considered valid if the male touched both silk threads with the front legs before he moved onto one of the threads and entered the attached web. The trial ended if the male started courtship. In *A. bruennichi*, courtship will generally result in mating (Zimmer and Schneider, unpublished manuscript). The position of the female categories was alternated with every trial. Each female was used only once in each state. We conducted three experiments that are

explained below. Males in experiment I and IIa were used once, while males were tested repeatedly in experiment IIb. Mated males were mated to a randomly selected female and the copulation was interrupted after 5 sec with the aid of a pointed spurt from a water pistol. Thereby we ensured that males survived copulation and were mostly unharmed. Males that had lost one or more legs during copulation were discarded.

#### Experiment I (state-dependent mate choice)

In the first experiment, males differed in their mating status (virgin or once mated) and in their age (young or old). The design was balanced with 14 males for each combination out of a total of 56 males. Age was measured in days elapsed since maturation and males were classified as young if less than 14 days had passed since maturation and they were considered old if more than 14 days had passed. The division was arbitrary and only dictated by the total number of males that was divided in halves. However, the cut-off at an age of 14 days makes sense as the selection regime for males will likely change in nature around this age. Males mature before females and it will take about one week until the majority of females in a population are mature (Zimmer et al. 2012). Hence, early males will experience strong competition for many virgin females while males older than 14 days will face mostly mated females and reduced competition.

Males could choose between a virgin and a mated female, each of which was in the right position in 50% of 56 trials. Each female was used only once. Females were weighed one day after maturation and the pairs were assorted such that one female was heavier than the other. However, there was no systematic difference in body mass of right (66mg, SD=19.3) and left (70mg, SD=20.9) females (t-test:  $t=-1.05$ ,  $P=0.30$ ) nor between virgin (69mg, SD=21.3) and mated (67mg, SD=19.1) females (t-test:  $t=0.59$ ,  $P=0.56$ ). Males chose the right female in 53% of the cases, which is not significantly different from a 50:50 distribution (likelihood-ratio:  $\chi^2=0.29$ ,  $P=0.59$ ). Therefore we use “right female chosen” as the dependent variable and the categories “virgin/mated”, “heavier/lighter” as factors in the statistical analyses.

#### Experiment IIa (context-dependent choice)

In the second experiment, males again differed in their **mating status**: 23 virgin and 23 mated males were given a choice between a subadult and a virgin adult female. The virgin female was presented on the right side in 21 trials and on the left side in 25 trials. Males chose the right female in 47% of the 46 cases. This is not significantly different from a 50:50 distribution (likelihood-ratio:  $\chi^2=0.09$ ,  $P=0.79$ ). Therefore, “right female chosen” was used as the dependent variable as above. Female body mass was determined at the day of the experiment for subadults and one day after maturation for adults and did not differ between right (74.5mg, SD=18.2) and left (74mg, SD=20.0) females (t-test:  $t=-0.02$ ,  $P=0.99$ ) but between virgin (67mg, SD=17.6) and subadult (83mg, SD=17.0) females (t-test:  $t=-4.34$ ,  $P=0.0001$ ). Therefore, the difference of body mass between the two females was used as a covariate in the statistical model.

The **context** was varied by presenting cues from a rival with one of the two females in 24 of the trials. The rival male walked along the left silk thread before the right one was attached to the skewer. Thereby a competitor was present in 11 webs of subadult females and in 13 webs of adult virgins. Rivals leave cues behind in the form of dragline silk that they always release and which will run along the thread from the female web. Rivals were virgin males without pedipalps. For the removal of the pedipalps we induced autotomy by gently squeezing the femur of the pedipalp with fine forceps until the male released the extremity. This method has been applied successfully before and does not influence male motivation to court or copulate (Schneider and Lesmono 2009). Autotomy of legs is a general adaptation of spiders and often used by males of sexually cannibalistic spiders to escape a female attack. By inducing autotomy, liquid loss is minimized. We produced five eunuch males that we re-used for all experiments.

#### Experiment IIb (repeated tests)

After we found that males did not discriminate very young virgins from subadult females, we added another experiment performed in 2012 (N=10) and 2013 (N=22). We tested males repeatedly to determine whether the indifference resulted from the behavior of all



males or whether similar proportions of males favored either subadult or virgin females. We tested these alternative hypotheses by testing each male three times. Each male encountered three different pairs of subadult and virgin females. The final molt to maturity was never more than 3 days ago in all virgin females. Weight differences were equally distributed among the females presented on the right side (mean=123.33 mg, SD=38.9) and on the left side (mean=117.81 mg, SD=35.7). Repeated trials were mostly conducted within two consecutive days with at least one hour between those trials that occurred on the same day. One male in 2012 and four males in 2013 were tested on the same day, 6 males in 2013 over a period of three days. Each male was virgin and trials were terminated as soon as males had reached the web of one of the females, in order to prevent copulation.

Unlike in the previous experiments, males tended to visit the female on the right side more often than the female on the left, however, the bias was not significant. During the first test round, males chose the right female 19 times and the left one 13 times (likelihood-ratio:  $\chi^2=1.13$ ,  $P=0.29$ ). In the other two rounds, the bias was 21:11 (likelihood-ratio:  $\chi^2=3.18$ ,  $P=0.07$ ) and again 19:13. Males were not consistent in their choices of right or left females (link scale repeatability:  $r=0.027$ ,  $P=0.416$ ; CI=[0, 0.279]).

### Statistical analyses

Most results were analyzed using logistic models in JMP 7.0.2 (2007 SAS Institute). Repeated tests were analyzed and repeatability assessed using generalized linear mixed models (GLMM) with binomial errors in R (R Development Core Team 2012). P-values for repeatability were obtained as link scale repeatability from multiplicative over-dispersion models according to Nakagawa and Schielzeth (2010).

## Results

### Experiment I: state-dependent mate choice

A full logistic model explained 53% of the variance and contained three interactions (Table 1). Removal of the interaction between male mating status and female weight (*Right female heavier*) did not change the explanatory

power much ( $r^2$  from 0.527 to 0.526). Removal of the interaction between *Right female virgin/mated* \* *Right female heavier* reduced the explanatory power of the model ( $r^2=0.49$ ). Therefore, we retained this interaction and did not further simplify the model (Table 1). The final model showed that most of the choices were explained by the mating status of the female and by the interaction of male age class and which female was the heavier one. Males significantly preferred virgin over single-mated females (ratio virgin:mated 43:13;  $\chi^2=16.95$ ,  $P=0.0001$ ), as expected. In addition to female mating status, female weight played a role as well in the interaction with male age. Old males selected the heavier female in 24 of 28 cases and young males in only 10 of 28 cases ( $\chi^2=15.58$ ,  $p=0.0001$ ).

Curiously, young males (N=28) were quicker during the choice procedure and they were found in the web of a female after a mean of 14.35 sec (SE=4.9) while old males (N=26) took 32.89 sec (SE=4.9) (t-test:  $t=-3.11$ ,  $P=0.003$ ).

**Table 1.** Results of a multivariate logistic model on the probability that males selected the female on the right side. One non-significant interaction was removed. The whole model is significant and explains 53% of the variation.

Factor	DF	L-R $\chi^2$	P-value
Right female virgin / mated	1	16.71	<.0001
Right female heavier	1	5.60	0.0180
Male virgin / mated	1	0.52	0.4706
Male young / old	1	6.76	0.0093
Male young / old * Right female heavier	1	18.59	<.0001
Right female virgin/mated * Right female heavier	1	3.08	0.0794
Male virgin / mated * Right female heavier	1	0.11	0.7439

### Experiment IIa: context-dependent choice

Males were given a binary choice between a virgin and a subadult female. In half of the trials the left female had been visited by a male before (treatment: *competitor*). The presence of a rival in the left web was nearly balanced between the two female states (13 subadults, 11 virgins) but had no effect on the probability that the male selected the right or

left female. In those trials in which a competitor was in the left web (N=24), the right female was chosen in 9 cases and the left one in 15 cases.

A logistic model with *right female chosen* as dependent variable that contained the factors: male mating status, female state (subadult or virgin), competitor and their interactions, as well as the size difference between the two females as covariate, was not significant (logistic model:  $\chi^2_8 = 5.59$ ,  $P=0.69$ ). None of the variables explained a significant part of the variation. Removal of the interactions did not improve the model much (logistic model:  $\chi^2_4 = 4.65$ ,  $P=0.33$ ) nor did a further stepwise removal of the least significant factors produce a significant model.

Subadult females were chosen 17 times (37%) and virgin females 29 times (63%), a difference that was not statistically significant (likelihood-ratio:  $\chi^2 = 3.17$ ,  $P=0.08$ ) but tended towards a preference for virgins. We inspected the data in more detail and found very similar frequencies of choices related to male mating status, female mass differences, and presence of cues of a rival (Table 2).

**Table 2.** Frequencies of choices between virgin and subadult females as a function of male mating status, female state and the presence or absence of a competitor.

Frequency chosen	Virgin female	Subadult female
Male virgin	14	15
Male mated	9	8
Virgin was heavier*	9	3
Subadult was heavier	17	13
Competitor present	14	10
Competitor absent	15	7

\*missing values in three pairs

The attractiveness of subadult females may be determined by how close maturation is, and the attractiveness or the signaling intensity of virgin females may depend on how close she is to oviposition. We counted the days until maturation in subadults and tested whether distance to maturity predicted the probability of the subadult female being chosen, but found no significant correlation (logistic regression:  $\chi^2 = 0.04$ ,  $P=0.85$ ). A logistic regression between

the number of days since maturation and whether the virgin was chosen or not was also non-significant ( $\chi^2 = 1.59$ ,  $P=0.21$ ). However, the distribution of the choice data suggested that above an age of 3 days past maturation, virgin females (N=11) were given preference in all but one case which was statistically significant (likelihood-ratio:  $\chi^2 = 8.55$ ,  $P=0.004$ ). While in the remaining 35 trials, virgins were selected 19 times and subadults 16 times (likelihood-ratio:  $\chi^2 = 0.26$ ,  $P=0.61$ ). Experiment IIb explored these patterns further.

### Experiment IIb: repeated choice tests

Virgin males were given a choice between a subadult and a young virgin (<3 days since maturation). Each male was tested three times on two days or rarely the same or on three days. The experiment was repeated over two years (2012: N=10 males; 2013: N=22 males). Males showed no preference in their first trial (16 males chose the virgin: 16 males chose the subadult). However, in the 2<sup>nd</sup> and 3<sup>rd</sup> trial a pronounced preference of virgin over subadult females emerged (2<sup>nd</sup> trial: 24 males selected the virgin: 8 the subadult; 3<sup>rd</sup> trial: 25 males selected the virgin: 7 the subadult). Accordingly, a GLMM with binomial error structure and male ID as random effect revealed that preferences significantly changed with trial number ( $\chi^2_2 = 6.52$ ,  $P=0.038$ ). However, males were not consistent in their choices (link scale repeatability:  $r=0$ ,  $P=0.803$ ;  $CI = [0, 0.278]$ ).

### Discussion

Our results confirmed that males of *Argiope bruennichi* have a strong preference for virgin over once-mated females, which is adaptive due to first male sperm precedence. However, age of males changed the main preference criterion from female mating status to female body weight. Their own mating state and the competitive context were not relevant for males in our setting. Males preferred virgins over subadults if maturation of the former had occurred at least three days ago. Younger virgins and subadult females were not discriminated during a first encounter, suggesting that males cannot detect female state per se but rely on signals or cues emitted by the female. Curiously, a preference for virgins emerged in subsequent encounters

without individual males being consistent in their choices.

Male state variables, mating status and age influenced mate choice preferences but were more complex than expected. Virgin and mated males showed the same strong preference for virgin females, which conflicts with model predictions (Fromhage and Schneider 2012) and with empirical findings from a congener (Gaskett et al. 2004). A model that was based on the biology of *A. bruennichi* predicted that mated males should show a stronger selectivity than virgin females (Fromhage and Schneider 2012). The model result was based on the fact that once-mated males invariably die during their second copulation, which will increase opportunity costs. This was not apparent in our set-up offering a simultaneous, hence less natural choice situation. The incongruence may not be surprising as it is adaptive for all males to reject a mated female if a virgin is within reach and can be picked without sampling costs. Mated males also suffer a higher risk of a mismatched choice as they have only one pedipalp available and due to the fixed insemination pattern in *Argiope*, a male can only achieve paternity success if the virgin spermatheca of the once-mated female matches his unused pedipalp. The used side will most likely contain a mating plug that will protect the sperm from the previous male (Nessler et al. 2007; Herberstein et al. 2012). In the Australian congener *A. keyserlingi*, males do distinguish once-mated from double-mated females but they cannot detect which of two once-mated females has the matching side unplugged (Zimmer et al. 2014).

An earlier binary choice study in *A. keyserlingi* found that mated males did not show a preference for virgin over mated females (Gaskett et al. 2004) but the results may be explained by sensory impairment of mated males. Males often lose legs during copulation and since legs are important for sensory input, mated males may no longer perceive the differences in chemical signature of virgin versus mated females. We controlled for male sensory acuity by only using mated males that still had all their legs. We also controlled for the confounding factor *age* in our experiment, by making sure that mated males were not older than virgin males. This allowed us to test the influence of age, independent of the influence of mating status. Interestingly,

age did alter male preferences. Older males paid more attention to female weight than to her mating status, while younger males preferred virgin over mated females even if the latter were heavier. This state-dependent change may reflect differences in expected fitness returns early and late in the season.

Females put on weight as egg development progresses and fat females are likely very close to oviposition. Many studies have demonstrated male preferences for fecund females in spiders and insects (e.g. Bonduriansky 2001; Danielson-Francois et al. 2002; Saeki et al. 2005; Barry et al. 2010; Schulte et al. 2010; MacLeod and Andrade 2014) but preferences for virgins tend to be stronger (Rittschof 2011).

An old *A. bruennichi* male can expect to fertilize at least 50% of the eggs of a mated female if he injects the entire sperm load of a pedipalp. Mated males always do this and virgin males can opt for a single but long first copulation (monogyny type 1; Fromhage and Schneider 2012; Welke et al. 2012). These estimates are based on double mating studies, in which relative paternity was found to positively correlate with relative copulation duration but only if two males inseminated different spermathecae (Schneider et al. 2006; Schneider and Lesmono 2009). Males that copulate into a used spermatheca can only expect to gain a paternity share in the rare occasion that the previous male failed to apply a mating plug (Nessler et al. 2007). Female weight generally predicts fecundity in spiders and the variation can be large enough to make a true difference to male reproductive success. Fertilizing 50% of the eggs of a very fecund female is not much different from fertilizing 100% of a female from the other end of the spectrum and weights of females in our experiment varied between 13 to 125 mg. The potential gain in the number of fertilized eggs may be further enhanced by the increased probability that the heavy female will survive until egg-laying as there are fewer days to go (Rittschof 2011). Younger females that will still need weeks to accumulate the resources for egg-laying may promise fewer gains at a higher risk once the season has progressed. Males of *A. bruennichi* are adapted to a very short mating season of three to four weeks and a male that is older than two weeks should opt for strategies that maximize reproductive success towards the end of the season when

fewer virgin females are around. Late in the season, imminent oviposition may entail the twofold advantage of increased likelihood of egg-laying plus all future benefits associated with timely production of offspring. For example, eggs laid too late in the season might fail to reach the appropriate developmental stage before winter.

*A. bruennichi* males are protandrous ensuring that most males encounter females that matured very recently. Hence, young males usually anticipate maturation of the majority of high quality females and the potential to monopolize paternity with one of these females may be the best way to maximize fitness. In addition, female weight may be less informative than for example body size under early conditions (note that size is based on sclerotized body parts and is fixed within each instar). Large females can put on more weight than small females. In addition, preferences for fecund females at this stage may be less beneficial due to increased competition over such females (Kokko and Johnstone 2002). These interpretations require further experimental verification.

Whether or not a rival had previously entered the web of a female did not influence decisions of males regardless of their mating state. This is unlike in *Nephila senegalensis*, another spider with a similar mating system, in which males have been found to avoid competition (Schneider et al. 2011) in a similar laboratory setting. Likewise, a study using the Australian *N. plumipes* found that males tend to avoid competitive situations when the context changes (Jordan et al. 2014) and in *Zygiella x-notata* males vary their mating preference with their competitive ability (competitive males mate with larger, fecund females; smaller males chose smaller females when competition is strong) (Bel-Venner et al. 2008). Male *A. bruennichi* are known to perceive direct competition as they have been shown to abbreviate courtship in the presence of a rival (Schneider and Lesmono 2009). It is unclear why males did not react to competition in our choice experiment. Perhaps the mere presence of rival silk at the point of choice in our setup was not a very reliable indicator of competition as this rival may have long moved on. Males may realize rival presence only when they enter the web and perceive movement. However, as long as the female is attractive

this will result in scramble competition between the competitors and each male will try to mate first (Schneider and Lesmono 2009). Males may also use draglines of other males to follow them if this increases the probability of finding a mate (Anderson and Morse 2001).

Several species although mostly fish have been tested in context-dependent mate choice experiments and the results are inconclusive (Callander et al. 2012). Presumably, fitness returns of context-dependent choice will depend on gains and more studies are required to search for general patterns. It remains to be seen in *A. bruennichi* whether male age alters responses to the competitive context.

In our second experiment, males did at first sight not show a preference for virgin over subadult females. However, a closer inspection revealed a pattern in the data suggesting a female age effect. If virgin females below a post-maturity age of 3 days were excluded from the analysis, a very clear preference for virgins became apparent. There are several possible explanations for this observation. A functional explanation could be that males have no need to distinguish virgin from subadult females because both provide very similar fitness returns. Subadults can be guarded until they mature and then monopolized (see below; Uhl, Zimmer, Renner, Schneider, unpublished). Alternatively, females may start emitting pheromones only a few days after maturation and males may require the pheromone to recognize virgins. Chemical analyses of the silk and bodies of females revealed that virgins produced the pheromone while subadults and mated females did not (Chinta et al. 2010). The explanation that young females do not emit pheromones is supported by recent field experiments showing that very young virgins do not attract males (Cory and Schneider, unpublished). This finding implies that those males that are regularly found near and in the webs of such females (Zimmer et al. 2012) must have stumbled over these webs by coincidence. Indeed, old females were found to attract most males, supporting the hypothesis that females use pheromone emission strategically (Cory and Schneider, unpublished).

Our results did not support the idea that a proportion of males seek young virgins while

another part of the male population opts to gain so called “soft matings” or “opportunistic copulations” (Foellmer and Fairbairn 2005). Males of the American *A. aurantia* can be regularly observed to engage in opportunistic matings. The same strategy has been identified as an alternative route to fitness for *A. bruennichi* males, as monopolization potential is indeed higher when mating with a soft female than when mating with a hardened female (Uhl, Zimmer, Renner, Schneider, unpublished). Indeed, during soft matings males never die during their first copulation and can deliver their entire sperm load, very much unlike males mating with virgins, in which up to 80% of the males will be killed and consumed during their first copulation (Schneider et al. 2006).

The existence of a matched polymorphism in preferences for soft versus hard copulations would have required that males are consistent in their choices. Exposure of males to three pairs of subadult and young virgin females in succession did not reveal individual consistency. In contrast, a significant preference for young virgins emerged during 2<sup>nd</sup> and 3<sup>rd</sup> trials. The ability or the necessity to recognize a mature and virgin female may change with experience. However, results are curious because individuals that chose the virgin in the 2<sup>nd</sup> trial did not necessarily make the same choice in the 3<sup>rd</sup> trial. In this last experiment, males showed a strong bias to the right side, which was, however, not consistent within individuals. We have no explanation for this bias as we controlled for uniform illumination and airflow. Furthermore, we excluded external disturbances by locking the experimental room. Female influences were unlikely as each female was used only once. The unintended bias may have had an influence on the above inconclusive findings. More experiments are needed to elucidate whether experience could have produced the observed pattern.

Spiders are generally known to be very responsive to cues on silk. Wolf spiders have been shown to differentially respond to silk cues from virgin and mated females (Roberts and Uetz 2005), from satiated, cannibalistic and hungry females (Moskalik and Uetz 2011), and even from females of different age classes that are known to be more or less receptive and potentially dangerous for males (Roberts

and Uetz 2005). Male *Schizocosa ocreata* were also found to differ in their reactions (courtship investment) to female cues during their first and second encounter (Moskalik and Uetz 2011). Differences can be accounted to experience with certain cues that change decisions in a subsequent situation and spiders are known to alter mate choice depending on experience as juveniles (Wilgers and Hebets 2012). Perception mechanisms and decision rules remain enigmatic and need to be studied further. We have no comprehensive concept of how spiders use environmental cues for decision making. Since spiders do show intriguing and tractable behavioral flexibility, they are ideal study objects for this field.

In summary, we could demonstrate state-dependent mate preferences of males although the competitive context had no influence. Nevertheless, assessment and decision rules evolved suggesting that they are favored by selection. Our results add to the growing body of evidence for male mate choice as a result of breeding investment.

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## Chapter 6

### Can males detect the strength of sperm competition and presence of genital plugs during mate choice?

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Female reproductive status can have strong selective effects on male mating strategies due to the threat of sperm competition, which may explain why males prefer virgin over mated females. However, in mating systems with female multiple mating rates and mating plugs, males should not only respond to the risk but also to the level of sperm competition and should be sensitive to the interference from mating plugs. In the orb-weaver spider *Argiope keyserlingi*, females possess paired sperm-storage organs facilitating separate sperm storage from different males. Males are limited to 2 copulations due to mutilation of their paired genitals (pedipalps). By conducting binary choice experiments, we tested whether males of different mating status can distinguish between females that mated with 1 or 2 males and whether single-mated males discriminate between single-mated females with matched or unmatched virgin genital openings. Furthermore, we investigated whether males adapt their mating strategies to the intensity of sperm competition by providing males with varying qualities of females in their immediate vicinity. Our results demonstrated that males are sensitive to the level of sperm competition and preferred single-mated females over double-mated females. However, they failed to identify single-mated females whose virgin genital opening matched their unused pedipalp, which is required due to their fixed ipsilateral insemination. Surprisingly, males never mated twice with the same female independently of the quality of surrounding females. This suggests that the benefits from searching and mating with a different female are greater than the benefits of monopolizing the female by mating twice with her.

**Keywords:** *Argiope*, mating plug, mating strategy, sex pheromone, spider

#### Introduction

Sperm competition, defined as the competition between ejaculates of males for the fertilization of the female's ova (Parker 1970), is a strong selective agent on male behavioral (Sawada 1999; Bretman et al. 2009), morphological (Miller 1990; Robinson and Novak 1997) and physiological traits (Andersson et al. 2000; Wedell 2005; Yamane 2013). Males are very sensitive to detecting the threat of sperm competition by assessing, for instance, the operational sex ratio (Clark 1988; Jablonski and Vepsäläinen 1995; Allen et al. 2011) or female mating status using olfactory (Schiestl and Ayasse 2000; Gaskett et al. 2004; Uhl and Rivera 2005; Barry et al. 2010) or visual cues (Orr and Rutowski 1991). However, our understanding of the fitness costs of sperm competition is largely based on a dichotomous approach, namely how many eggs does males fertilize if he has to share paternity with a single other male compared with monopolizing

the female (Lewis and Austad 1990; Tsubaki and Yamagishi 1991; Simmons and Siva-Jothy 1998; Simmons 2001). Few studies have looked at sperm competition patterns extending this classic dichotomy to situations when 3 or more males are involved (Elgar et al. 2003; Bonilla et al. 2011). Those studies have found that fitness costs are greater for a third male than for the second male (Bonilla et al. 2011). Consequently, fitness costs are likely to vary with the number of males a female has mated with, and we expect that males are sensitive not only to whether a female has mated or not, but also to the number of males she has mated with (Bonilla et al. 2011).

Not surprisingly, the dichotomous approach to sperm competition is also applied in the context of genital plugs where an “all or nothing mechanism” is often assumed (Masumoto 1993; Fromhage and Schneider 2006; Uhl and Busch 2009). Mated females are plugged by male secretions or fragments of male genitals and are hence inaccessible for

subsequent males. In some cases, however, males can still copulate even in the presence of genital plugs, but there may be a reduction in fertilization success (Koeniger 1991; Moreira and Birkhead 2004; Snow et al. 2006). To complicate things even further, in some species, including all entelegyne spider species (Foelix 2011), females have multiple genital openings and the presence of a plug in one of them does not preclude copulation and sperm transfer into the unused and unplugged genital opening as shown in the redback species *Latrodectus hasselti* (Snow and Andrade 2005). Thus, male responses to mated females in systems with genital plugs may be sensitive to how likely the plug will interfere with successful sperm transfer and hence fertilization success. Moreover, the use of male genital fragments as a genital plug, which is a paternity protection mechanism that has evolved several times independently in spiders (Miller 2007), comes at a cost for males because the paired sperm transferring organs (pedipalps) can be used only once due to the damage (one-shot genitalia) (Fromhage and Schneider 2006). This mating strategy restricts males to maximally 2 copulations, one with each pedipalp (for exceptions see Snow et al. 2006).

In entelegyne spiders (e.g. orb-web spiders), females have paired sperm-storage organs (spermathecae) that connect to the outside via independent insemination ducts and genital openings (Eberhard 2004; Foelix 2011; Herberstein et al. 2011). In orb-web spiders, males empty 1 pedipalp into 1 spermatheca and often return to use the second pedipalp to inseminate the other spermatheca (Foelix 2011). This pattern is particularly rigid in the genus *Argiope* (Araneidae), where external female morphology together with very complex morphology of the male genital apparatus only permits a strictly ipsilateral insemination pattern (Uhl et al. 2007). Thus, males can only use the right pedipalp to insert into the female's right genital opening and the left pedipalp into female's left genital opening. In order to fully monopolize the female, males must therefore pursue a monogynous strategy and achieve 2 copulations with the same female, plugging both of her copulatory ducts. The evolution and maintenance of such a monogynous strategy, which is not uncommon in spiders, will depend on the efficacy of

paternity protection strategies (Fromhage et al. 2005) such as mate plugging.

Nevertheless, not all males achieve both copulations with the same female and thus forfeit 50% of the paternity success if the female mates again with a male who fills the other, still virgin spermatheca. This may be due to the prevalence of sexual cannibalism during the male's first copulation (Elgar et al. 2000; Foellmer and Fairbairn 2004; Schneider et al. 2006). But even in the absence of sexual cannibalism, males do not always complete their second mating with the same female. These bigynous males move to another female for their final copulation. In the genus *Argiope*, for instance, species vary in the frequencies of mono- versus bigynous males and whether or not these strategies are conditional or not. In *Argiope aurantia*, monogyny is seemingly obligatory with surviving males always completing their second copulation with the same female (Foellmer 2008), whereas in *Argiope bruennichi*, males follow a bigynous strategy when their first mate is relatively small with limited fecundity potential, but are monogynous if their first mate is large (Welke et al. 2012). By contrast, in the Australian St Andrew's Cross spider *Argiope keyserlingi*, males were never observed to mate twice with the same female (Herberstein et al. 2005). However, if the male leaves his first mate, he faces the risk of encountering a single-mated female where her virgin copulatory duct does not match his unused pedipalp or a double-mated female with both copulatory openings plugged.

The *Argiope* mating system extends the classic sperm competition paradigm because not only female mating status but also the number of matings a female has previously achieved will have significant fitness costs for males. We know from previous study that *A. keyserlingi* males distinguish between females of different reproductive status using airborne and silk-based sex pheromones (Herberstein et al. 2002; Gaskett et al. 2004). Here we extend this work asking how sensitive are males to not only the threat of sperm competition but also the level of sperm competition and whether this affects the decision to complete both copulations with the same female (monogyny) or seek a second mate (bigyny). We predict that *A. keyserlingi* males prefer single-mated over double-mated females. Furthermore, even

single-mated females may be undesirable for single-mated males if the only compatible copulatory opening for his unused pedipalp is already plugged. Based on previous observations of male behavior with single-mated females (Fromhage and Schneider 2005; Herberstein et al. 2005), however, we predict that males are unable to detect which copulatory opening of the female is plugged when he enters the web. Finally, we predict that males will pursue a monogynous mating strategy if available females are mated and carry a high risk of being incompatible and a bigynous mating strategy if the available females are unmated.

## Materials and Methods

### Study species and study site

The orb-web spider *A. keyserlingi* occurs along the East coast of Australia in high densities in urban parks, particularly in *Lomandra* sp. bushes (Herberstein and Fleisch 2003). The females are much bigger than the males and are easy to detect by their noticeable striped pattern on the abdomen and the bright silken decoration forming a cross in the centre of their web. We collected sub-adult *A. keyserlingi* females and males from 3 different urban parks in Sydney in November 2011 (West Pymble Park, Hughes Park, and Leichhardt Park).

### Laboratory maintenance

All individuals were raised to adulthood in the laboratory of Macquarie University, Sydney. Males were kept in individual upturned plastic cups of 250 ml, whereas females were housed in 330 ml plastic cups. All spiders were watered and checked for moults daily to determine the exact date of maturation. Males were fed with approximately 15-20 *Drosophila* sp. and females received 3 Queensland fruit flies *Bactrocera tryoni* (Diptera) twice per week. After females moulted to maturity, they were transferred to individual large Perspex frames (58 × 58 × 10 cm) in which they built their typical orb-web overnight, and they were used 24 h later in experiments. Males stayed in plastic cups until the experiments began.

In the laboratory, we weighed all spiders on their day of maturation and the day of experimentation. Males were weighed before

trials, whereas females were weighed afterwards to avoid damage to their orb-webs prior to trials. Weight was measured in milligrams on an electronic balance (Mettler Toledo New Classic MS). The mean weight of males was  $16.25 \pm 0.42$  mg (N=53), and the mean weight of females was  $160.66 \pm 3.66$  mg (N=105) at the day of the trial. Additionally, we measured the tibia-patella length of the first pair of legs with digital callipers as an indicator of body size. The size was measured in millimeters and the measurements were taken after the experiment to avoid excessively handling the spiders before the trial. The mean leg length of males was  $3.01 \pm 0.31$  mg (N=52) and the mean leg length of females was  $7.13 \pm 0.08$  mg (N=108). Sample sizes for weight and legs differed due to missing data.

### Influence of female quality on male mating strategy

In this experiment, we tested if males adapt their mating strategy to the availability of females of varying qualities in their immediate vicinity (see also observations by (Herberstein et al. 2005)). Female mating status is an important quality criterion for males as it directly affects potential paternity returns. Hence, we use quality as an equivalent for mating status. We predicted that males are monogynous and mate twice with the same female if any other available female carries the risk of sperm competition (i.e. mated females). Conversely, males are expected to switch to bigyny if the available females are unmated.

We set up a central mating pair, consisting of a virgin male to perform his first copulation with a virgin female. We surrounded the central mating pair with a circle of females (Figure 1). We used 23 virgin females and 24 single-mated females that were randomly assigned to groups of 4 for each trial. Although individual females were reused, the group formations were never used more than once. The circle females were on freshly built webs in Perspex frames (58 × 58 × 10 cm). We conducted 20 trials in which the central mating pair was surrounded either by 4 virgin females ( $N_{\text{trials}}=10$ ) or 4 single-mated females ( $N_{\text{trials}}=10$ ). We gently introduced the male into the corner of central female's web and recorded the duration of courtship and copulation, whether the male copulated once or twice with the female and whether he

stayed in female's web or moved to another available female. We set up fans on both sides of the experimental arena to ensure that the males received all female pheromones. All trials lasted for 3 h.

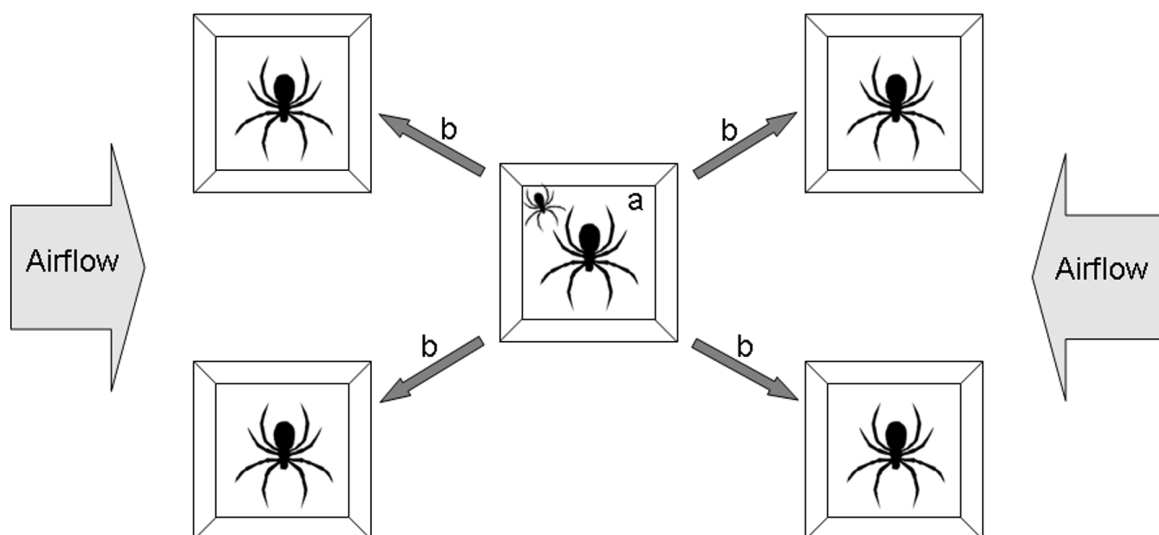
### Male mate choice

We conducted mate-choice experiments to test whether males can detect the difference and show a preference for single- over double-mated females. The quality of these females differs substantially. Not only is the intensity of sperm competition heightened in double-mated females, but also double-mated females likely have both copulatory openings plugged by their previous mates, preventing subsequent matings (Herberstein et al. 2012). Furthermore, we tested whether single-mated males show a preference towards single-mated females with the virgin genital opening compatible with his pedipalp availability. In other words, we tested whether males that had used their right (or left) pedipalp with another female, can distinguish between females that have their right hence matching (or left respectively) genital opening plugged. Such females would be just as invaluable for a male with a single available pedipalp as a double-mated female.

In the first choice experiment, virgin males had the opportunity to choose between a

single-mated female and a double-mated female. In the second experiment, single-mated males were offered a choice between a compatible single-mated and double-mated female. Here, the male's unused pedipalp was compatible with the female's unused genital opening. In the third experiment, single-mated males chose between pairs of compatible single-mated females and incompatible single-mated females.

For each trial, 2 frames containing females in their web were placed at an approximately 100° angle to each other. We dissected a silk thread from each of the 2 webs and carefully attached them to the tip of a wooden skewer. The wooden skewer was about 20 cm long and was fixed at an angle of around 30° to the bench top in front and between the 2 frames (see Gaskett et al. 2004). Trials began by gently placing the male with a paintbrush at the base of the wooden skewer. Usually, males walked along the skewer until they reached both threads. Trials were scored as successful and included in the analysis when the male touched both silk threads. We recorded the time from placing the male on the skewer until touching both silk threads and the time from touching both threads until making a decision for 1 of the 2 females, as well as the behavior during 10 min after the male made a choice.



**Figure 1.** Experimental arena for testing male responses to the varying qualities of nearby females. A central mating pair consisting of a virgin female and virgin male is surrounded by a circle of either 4 virgin females or 4 single-mated females on a table. All females sit in their webs built in frames. Fans indicated by big arrows on both sides of the experimental arena ensure that the males receive all airborne female pheromones. Males can either (a) mate twice with the central female (monogynous strategy) or (b) mate only once with the central female and move to another female in the vicinity (bigynous strategy).

Females and males were chosen randomly for each trial. However, the 2 females were approximately size matched. Females were never paired up more than once and males were never introduced to the same female twice. Mated females and males were used two days after copulation at the earliest, and all spiders had to be adult for at least 2 days before the experiments. Plug occurrence was predicted by the 100% frequency of breakage of pedipalps in *A. keyserlingi* males (Herberstein et al. 2012). However, the previous study also showed that plug efficiency is determined by copulation duration and the elapsed time from day of plugging (Herberstein et al. 2012). We ensured that male plugs were effective by allowing males long copulations (we prevented females from attacking the males by gently placing a paintbrush against her chelicerae which prevented her from biting the male) and by using mated females shortly after mating. Females containing females in their webs were placed randomly to either side of the choice apparatus in every trial to prevent a consistent position of females with the same mating status.

### Statistics

All analyses were performed using the statistical program JMP 7.0.2. Binomial tests and Fisher's Exact tests were used to analyze male preferences in the choice experiments. A logistic regression examined the influence of male mating status and female age on male's choice. A pairwise correlation was used to analyze data from the size-assortative choice experiment. Descriptive statistics are given as mean  $\pm$  standard error.

## Results

### Influence of female quality on male mating strategy

In all trials, the male mated with the central female (100%). The average time from trial start until mating was  $108.99 \pm 13.77$  min (N=20). Twelve of 20 males (60%) moved to the edge of the central female's web after mating and stayed at the edge without the attempt to copulate again within the 3-h trial. On average, males had  $73.75 \pm 12.85$  minutes (N=13) left to restart courtship within the 3-h trial. One male (5%) restarted courtship subsequent to his first

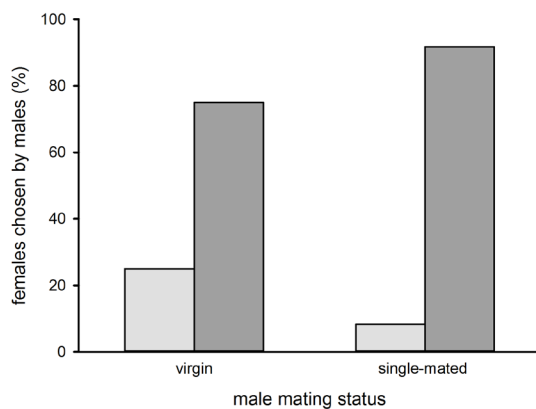
mating, copulated twice with the same female and was cannibalized by that female.

Seven of 20 males (35%) were cannibalized after the first mating, preventing them from further matings. The cannibalism rate was independent of the mating status of the surrounding females (nominal logistic regression:  $\chi^2_1=0.22$ ,  $P=0.64$ ,  $N=20$ ). No male that survived the first copulation moved to another available female. Male behavior following his initial mating with the central female was independent of the mating status of the surrounding females (nominal logistic regression:  $\chi^2_1=1.64$ ,  $P=0.2$ ,  $N=13$ ).

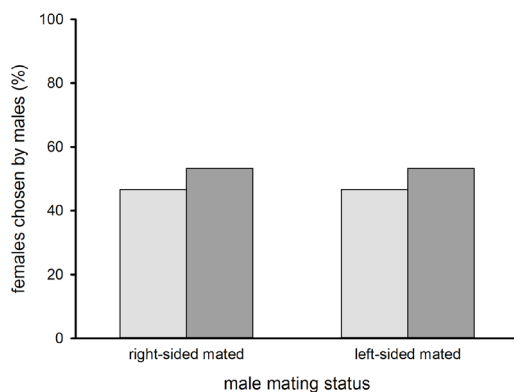
### Male mate choice

In total, 54 experiments involving male's mate choice were successful in which the male touched the threads of both webs and then walked along one of the thread and entered the web of the chosen female. On average, males made a choice after  $36.48 \pm 8.52$  s. Twenty four of the 54 males (44.4%) started to court after they reached the web, proceeded to the hub and touched the female. Six males (11.2%) moved straight to the female they had chosen and touched her shortly before moving to the edge of the web without courtship. The remaining 24 males (44.4%) moved immediately to the edge of the web they had chosen and stayed there motionless for the following 10 min until the end of the trial.

In the first 2 mate choice experiments, males chose single-mated females significantly more often than double-mated females (Binomial test:  $\chi^2=10.67$ ,  $P=0.001$ ; Figure 2) independently of their own mating status (logistic regression:  $\chi^2_1=1.25$ ,  $P=0.26$ ,  $N=24$ ) and the age of the females (logistic regression:  $\chi^2_1=0.09$ ,  $P=0.77$ ,  $N=24$ ). Nine of 12 virgin males (75%) preferred single-mated females over double-mated females, and 11 of 12 single-mated males (91.7%) chose single-mated females over double-mated females.



**Figure 2.** Summarized results of the first 2-mate choice experiments including the percentage of virgin and single-mated males that chose either double-mated females (light grey column;  $N_{\text{virgin}}=3$ ,  $N_{\text{mated}}=1$ ) or single-mated females (grey column;  $N_{\text{virgin}}=9$ ,  $N_{\text{mated}}=11$ ).



**Figure 3.** Percentage of single-mated males that chose either single-mated females that have virgin genital opening matched with their unused pedipalp (light grey column;  $N=14$ ) or not (grey column;  $N=16$ ) in the third mate-choice experiment.

However, in the third treatment, in which single-mated males ( $N=30$ ) had the opportunity to choose between single-mated females whose virgin genital opening either matched their unused pedipalp or not, we found no evidence of preference (Fisher's Exact test:  $P=1.0$ ). Seven of 15 males with an unused right pedipalp (46.7%) and 7 of 15 males with an unused left pedipalp (46.7%) chose the correct female whose unused genital opening matched with their unused pedipalp (see Figure 3).

Pooling the data from all 3 choice experiments, we found that males overall showed no apparatus side preferences

(Binomial test:  $\chi^2=1.19$ ,  $P=0.28$ ), and there was no evidence of a size-assortative choice as male body size was not correlated with the body size of the chosen female ( $r=-0.11$ ,  $N=52$ ,  $P=0.43$ ) nor did we detect a correlation between the body masses of the males and the chosen females ( $r=0.04$ ,  $N=52$ ,  $P=0.78$ ).

## Discussion

Our results demonstrate that *A. keyserlingi* males were able to distinguish between single- and double-mated females and that they showed a general preference for single-mated females independently of their own mating history (virgin or single mated). However, single-mated males did not discriminate between single-mated females with matched or unmatched virgin genital openings and failed to detect mated females that provided realistic chances of gaining paternity.

Male mate choice is predicted in scenarios when mating opportunities are limited by high reproductive costs and a large variation in the quality of available females is present (Gwynne 1991; Bonduriansky 2001). In addition, male mate choice can evolve under increased investment into mating effort (e.g. mate attraction or intrasexual competition for mates) independent of male investment into parental care (Edward and Chapman 2011). Many species of spiders fall into the former 2 categories, due to the limitations on male copulation frequency (e.g., cannibalism or genital damage) and the rapidly changing reproductive landscape over a season resulting in variation in female quality (Zimmer et al. 2012). Thus, it is not surprising that male mate choice has been broadly established in spiders (Riechert and Singer 1995; Gaskett et al. 2004; Andrade and Kasumovic 2005; Kasumovic et al. 2007; Schulte et al. 2010; Tuni and Berger-Tal 2012). What has not been known to date is how much information about female quality males can obtain. We clearly show that males can distinguish between a female who has mated once or mated twice, independent of her age. The fitness implications of this preference are clear: Not only are double-mated females likely to be inaccessible if successfully plugged, but even if the male can bypass the plug, his paternity is likely much less as a third male compared with being the second male. While we do not have paternity estimates for more

than 2 males in *Argiope*, in the orb-web spider *Nephila plumipes*, first males fertilize around 45% of eggs, but third males only obtain 23% of fertilizations (Elgar et al. 2003). This pattern is similar to other nonspider systems (Zeh and Zeh 1994).

Despite previous studies on spider pheromone cues (Gaskett 2007), it is still unclear what specific cues males are using to distinguish between once and twice mated females and thus to determine the sperm competition intensity. In some butterflies the male spermatophore is externally visible and thus could be used as a visual cue (Dickinson and Rutowski 1989; Orr and Rutowski 1991; Orr 2002). However, using visual cues to assess the level of sperm competition is unlikely in orb-web spiders due to their poor vision. As in our study and many other studies (Andrade and Kasumovic 2005; Roberts and Uetz 2005; Stoltz et al. 2007), male preference was tested using female silk; hence, some chemical cue is likely to convey this information. The interesting question is whether males transmit these cues during mating (see Baer et al. 2001; Wedell 2005) and the more males a female mates with the stronger the cues, such as in crickets (Thomas and Simmons 2009) or whether the emission of chemical cues is under female control and they change their pheromone signature by degrees. This may occur if mated females have no interest in attracting additional suitors due to the costs associated with the presence of males on their webs (Herberstein et al. 2002). Consequently, males may receive only pheromones from the silk threads of single-mated females, which would explain their preference in this experiment. However, we just do not know enough about *Argiope* pheromones to generate more specific predictions about the cues that enable males to determine precisely with how many males a female has copulated.

Although we showed that males distinguish between single-mated and double-mated females, they have limited sensitivity to the likelihood of interference from mating plugs when offered females with matched or unmatched virgin genital openings. This is in accordance with other studies on *A. keyserlingi* (Herberstein et al. 2005) and the congener *A. bruennichi* (Fromhage and Schneider 2005). Different results were found in *A. aurantia* in which the majority of males were able to avoid

plugged copulatory ducts (Foellmer 2008) when they were allowed to court females and probe their ducts. Similar findings are reported from the Australian redback spider *L. hasselti* (Snow and Andrade 2005) in which males preferentially inserted into the virgin genital tract after they contacted the female genital region. Male redback spiders perform an extensive and long-lasting courtship on the female's abdomen (Stoltz et al. 2008). Through this body contact males may collect information about existing mating plugs. Another study on the golden orb-web spider *Nephila edulis* that showed male's ability to detect female virgin genital openings despite the absence of mating plugs discussed the drumming behavior of male pedipalps on female's abdomen prior mating as one reason for cue perception (Jones and Elgar 2008). In our experiment, we restricted information to cues on the silk alone, which may have hampered male mate choice. However, body contact between *A. keyserlingi* females and males before copulation is limited to the male touching the female's leg and abdomen with his first and second pair of legs during courtship (Wignall and Herberstein 2013), which may not be sufficient to collect the relevant cues. Nevertheless, due to the restriction in cue perception in our experiment, we cannot rule out the possibility that males can extract information about which side is plugged if they have time to collect cues from the female's body directly. Investigating whether *A. keyserlingi* males are able or not to detect a plugged genital opening would be an interesting next step. Indeed, *A. bruennichi* males were found to limit their insertion duration while copulating in plugged genital openings (Nessler et al. 2007).

Contrary to our predictions, males did not switch between monogyny and bigyny as shown for their congener *A. bruennichi* (Welke et al. 2012). Considering that *A. keyserlingi* males are able to perceive airborne female cues (Gaskett et al. 2004), we can safely assume that our experimental design was suitable for focal males to detect the presence of other females around the central female. However, we cannot exclude that the duration of the presentation of the surrounding females was too short for *A. keyserlingi* males to clearly assess the varying quality of nearby females and hence the different reproductive

landscape. As a previous study has shown that *A. keyserlingi* males remain at female webs without attempting to copulate again even after several hours of their first copulation and left to search for another mate after this period of mate guarding (Herberstein et al. 2005), we however consider it unlikely that our trial length would have affected the behavior of males.

An alternative explanation might be that *A. keyserlingi* males have no need to adjust their behavior flexibly because they can rely on stable and predictable conditions, which is different for their congener *A. bruennichi*. A stochastic dynamic game model (Fromhage and Schneider 2012) predicts that a monogynous strategy should occur most commonly with large, rare, and virgin females in the habitat, whereas bigyny is favoured with small and mated females and at the beginning of the mating season as well as under high population density (Fromhage and Schneider 2012). As these time- and context-dependent patterns match findings in *A. bruennichi* (Welke et al. 2012), the reproductive landscape might be different in *A. keyserlingi* explaining the predominant bigynous mating strategy. For example, *A. keyserlingi* females are smaller and less variable compared with their congener *A. bruennichi* (SM Zimmer, personal observation). Furthermore, the mating season for *A. keyserlingi* is longer and may provide time to find suitable mates, but also lead to a higher probability of encountering mated females. Therefore, *A. keyserlingi* males may obtain a higher average fitness through a bigynous strategy suggesting that bigyny does not appear to be part of a conditional mating strategy, but rather a fixed one in this species.

In summary, our study moves beyond the traditional dichotomous approach to sperm competition and demonstrates nuanced male mating strategies that not only take into consideration whether a female has mated but also with how many males she has mated. Nevertheless, males must still approach the female and attempt a mating to ascertain if her genital opening is blocked by a sperm plug. Given these limitations, we predict that in this species, virgin females are available throughout the mating season.

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## General discussion

The major aim of this thesis was to investigate whether genetic compatibility in the form of inbreeding avoidance is a selective force responsible for the evolution of mating strategies in monogynous mating systems of the spider genus *Argiope*. I showed that matings between relatives lead to severe inbreeding depression indicated by reduced hatching success. These costs of inbreeding might have promoted the evolution of inbreeding avoidance mechanisms, such as strategic polyandry and trade-up mate choices, which could be demonstrated in *Argiope* in this thesis. However, I found a high genetic diversity, but still a modest individual inbreeding risk within natural populations of *A. bruennichi* as relatives did occasionally sit in the vicinity and might encounter one another.

The second aim of this thesis was to investigate the evolutionary significance of male mating preferences in *Argiope*. I showed that males evolved different mating strategies to optimise their mating success and that these strategies were dependent on varying female quality and availability over the mating season, as well as on the presence of sperm competition.

### Sexually selected compatibility

#### Risk and costs of inbreeding

Mating between close relatives often leads to a decrease in individual's fitness (Charlesworth and Charlesworth 1987; Keller and Waller 2002), so selection to avoid or reduce these costs should be strong. Several avoidance mechanisms have been detected (Pusey and Wolf 1996) and post-copulatory mate choice for genetically compatible mating partners facilitated by polyandry is one of them (Tregenza and Wedell 2002; Simmons 2005). However, for such a selection pressure to be relevant, the possibility for inbreeding as well as the possibility to avoid it must be present. I found high genetic diversity (chapter 1, chapter 2) and no fine-scale genetic substructure (chapter 2) in natural populations of *Argiope bruennichi*. Contrary to my expectations, neighbouring spiders did not

show an increased genetic similarity compared to distant spiders (chapter 2). However, some relatives stayed in the proximity and faced a probability of encountering each other as mating partners. Thus, these individuals face the risk of inbred matings, although at a modest rate (chapter 2).

The observed high genetic diversity is in agreement with another study on *A. bruennichi* showing that the colonisation by this species occurs with relatively large numbers of individuals from several origins, which results in an admixture of different lineages (Krehenwinkel and Tautz 2013). Thereby the probability of finding a compatible mating partner is increased and is particularly advantageous in *A. bruennichi* that suffers from reduced hatching success already after one generation of inbreeding (chapter 1). The costs of inbreeding and the present natural risk of encountering siblings combined with the possibility of finding a compatible mate might select for inbreeding avoidance in this species (see chapter 3). However, pre-copulatory avoidance mechanisms seem to be absent in males of *A. bruennichi* as male rejection of some virgin females was not correlated with an increased genetic similarity between these potential mates (chapter 2). Further studies are necessary to assess potential other types of incompatibilities responsible for male negative attitudes.

#### Strategic multiple mating

Polyandry generates the opportunity of cryptic female choice, defined as a female-controlled paternity bias towards certain males (Eberhard 1996). A previous study has shown that *Argiope* females choose post-copulatorily and favour sperm of unrelated males after mating with a sibling and a non-sibling male to avoid inbreeding (Welke and Schneider 2009). As *Argiope* females are initially indiscriminate in their mate choice (see chapter 4), they should have a particularly strong interest in attracting another male after they had mated with a sibling (low quality mate) to receive additional matings and to bias paternity towards the superior male. In chapter 3, I showed that *A.*

*bruennichi* females adapted their mate attraction depending on the quality of the first mate. If females had mated with a sibling, they seem to strategically continue advertising via sex pheromones and consequently attract further males for additional matings. Instead, mated females that had received a mating with an unrelated male were less attractive for males. This suggests that the costs associated with mate attraction and pheromone production (Johansson and Jones 2007; Harari et al. 2011; Umbers et al. unpublished) are only outweighed by the benefits of polyandry if the first male was of low quality. This also indicates that mated females halt pheromone production if further mate attraction is not beneficial. The variation in pheromone production is in accordance with other studies on spiders (Baruffaldi et al. 2010; Baruffaldi and Costa 2014) and insects (Harari et al. 2011) that largely explains male discrimination among females of different reproductive value. However, to my knowledge this study is one of a few experimental studies (see Perampaladas et al. 2008) suggesting that pheromone production might be linked to the benefits of polyandry.

### Opportunistic mate-choice

Individuals should mate indiscriminately at the first mating opportunity if mates encounter each other only sequentially and a risk of remaining unmated exists, but should preferentially re-mate with mates of higher genetic quality (trade-up hypothesis, Halliday 1983; Jennions and Petrie 2000). In chapter 4, I found support for this trade-up mate choice in *Argiope lobata*. Females and males showed no discrimination at their first mating opportunity, independent of whether the mate was a sibling or a non-sibling. However, they were extremely reluctant to mate with a sibling during second mating opportunities. These results are consistent with mating strategies of other species (Johnsen et al. 2000; Foerster et al. 2003; Laloi et al. 2011). However, while the trade-up hypothesis postulates that mated individuals should only mate with an additional mating partner of higher quality than the first mate (Halliday 1983), I found no significant influence of the relatedness of the first mate, neither did the inbreeding history affect mating decisions in *A. lobata* (chapter 4). This suggests that this species rather follows a

general rule of avoiding sibling matings while accepting matings with compatible males after they have secured sperm for fertilisation, independent of whether the sperm was of low or high quality. Thereby, multiple mating will increase the reproductive success of both sexes as females can reduce the contribution of incompatible sperm from a first male via cryptic mate choice and males can compensate inbreeding costs of the first mating by choosing only superior females in successive matings. Altogether, this finding is a further indication for the evolution of polyandry as an inbreeding avoidance mechanism in the genus *Argiope*.

## Male mating strategies

### Context-and state-dependent mate choice

A theoretical model predicts that male mate choice can evolve in species with low mating rates if males encounter a large variation in female quality and availability over the mating season (Fromhage and Schneider 2012). According to the model, male mating preferences should be linked to the fecundity of females over the mating season. Furthermore, males should be choosier after having mated once because they always die after the second mating and male choosiness is predicted to decline towards the end of the season. In chapter 5, I showed that males of *Argiope bruennichi* made state-dependent mating decisions and based this on the timing of the mating season. In general, both virgin and mated males showed a strong preference for virgin females independent of their own mating state. This finding is in contrast to the model prediction (Fromhage and Schneider 2012). However, for all males it is highly adaptive to choose a virgin female due to first male sperm priority caused by mating plugs. In particular, mated males face a cost in addition to the presence of sperm competition when encountering a once-mated female; a mated male can only mate into a genital opening that matches his unused pedipalp due to the fixed insemination pattern (see also chapter 6) and this may be the case in only 50% of once-mated females.

With advancing age, males changed their mate choice criterion and rather preferred a fecund female, independent of whether she was virgin or mated (chapter 5). This state-

dependent change might be a result of variation in breeding success early and late in the season. In *A. bruennichi*, the mating season is very short with a large amount of virgin females in the beginning that exponentially decreases towards the end of the season (Zimmer et al. 2012). Hence, preferring virgin females in the beginning of the mating season is adaptive for males as they have still enough time left for the mate search and the chance to encounter virgin females is high. Instead, males might maximise their reproductive success with a heavier female, even if she is mated, in the end of the season where not many virgin females are left. Heavier females produce more eggs than lighter females and males can still sire half of the eggs of a once-mated female. Thus the fertilisation success is similar when mating with a more fecund but mated female compared to mating with a less fecund female. An additional benefit of mating with a heavy female is that these are often close to oviposition and hence likely survive until egg-laying (Rittschof 2011), which further enhances male mating success.

Furthermore, *A. bruennichi* males showed a preference for virgin over sub-adult females, but only when the maturation of virgin females were longer than 3 days ago (chapter 5). Virgin females produce sex pheromones to attract males, while sub-adults do not (Chinta et al. 2010). However, males seemed to be unable to differentiate between a young virgin female (< 3 days) and a sub-adult female (chapter 5). This indicates that the sex pheromones required for males to recognise them as virgins are only emitted a few days after maturation. The latter finding is further supported by a recent field study showing that very young females do not attract males (Cory and Schneider, unpublished).

Still, although not emitting sex-pheromones, sub-adult females are sometimes guarded by males until they mature and males copulate after the females have just moulted. This significantly reduces the risk of being cannibalised during the first copulation and allows males to monopolise the female (Uhl, Zimmer, Renner, Schneider, unpublished manuscript).

Overall, the competitive context was not relevant for male decisions in *A. bruennichi* which is in contrast to other spider species

(Bel-Venner et al. 2008; Schneider et al. 2011; Jordan et al. 2014) where males have been found to avoid competition. Additional work is needed to precisely assess how males receive cues indicating the strength of male-male competition.

### Mate choice in response to sperm competition

In *Argiope*, female mating status comprises the threat of sperm competition and the presence of genital plugs and hence should have a strong selective effect on male mating preferences. In chapter 6, I showed that *Argiope keyserlingi* males were able to notice the risk and also the intensity of sperm competition and preferred single-mated over double-mated females. This preference is highly adaptive as double-mated females likely have both of her genital openings plugged. Even if males are able to remove the plug, they can still expect a reduced paternity share as a third male due to sperm competition. However, I also showed that males cannot detect single-mated females whose virgin genital opening matched their unused pedipalp (chapter 6). This finding is in accordance with a previous study (Herberstein et al. 2005), but also differs from findings in other spider species showing that males are able to avoid plugged genital openings (Snow and Andrade 2005; Foellmer 2008). While in these contrary studies, males were able to collect cues from female's body, I limited males to perceive only cues from silk in the experiment. This may have hampered mate choice and hence required further investigations.

Contrary to my expectations, *A. keyserlingi* males did not adapt their mating strategies to the availability of females of varying qualities in their surrounding (chapter 6). Males never mated twice with the same female, even when the available females were mated and carried a risk of sperm competition and unmatched virgin genital openings. This suggests that the bigynous strategy is fixed in *A. keyserlingi* which is in contrast to the congener *Argiope bruennichi* where a conditional mating strategy is present (Welke et al. 2012). This is an interesting observation, pointing to an abundance of virgin females in natural populations, which is unknown for other species in the genus *Argiope*.

In conclusion, I was able to show that the mating strategies in monogynous mating systems of *Argiope* are likely shaped by the selection to avoid inbreeding. The occurrence of a modest inbreeding risk and the costs of inbreeding in nature might thereby be the driving forces. Furthermore, the evolution of male mate preferences in *Argiope* is likely driven by the variability of female quality and availability, as well as sperm competition.

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## Author contribution

### Chapter 1: Rapid range expansion is not restricted by inbreeding in a sexually cannibalistic spider

Jutta M. Schneider (JMS) conceived and designed the experiments. Stefanie M. Zimmer (SMZ) sampled the populations, performed the mating experiments, conducted the microsatellite analyses, and analysed all data. Henrik Krehenwinkel developed the markers and conducted the mitochondrial sequencing. The paper was written by SMZ with contributions from JMS.

### Chapter 2: The strength of sexual selection on inbreeding avoidance in natural populations of a spider

JMS conceived and designed the experiments. SMZ sampled the populations, performed the field experiments, conducted the microsatellite analyses, and analysed all data. The manuscript was written by SMZ with contributions from JMS.

### Chapter 3: Strategic polyandry in a sexually cannibalistic spider

JMS conceived and designed the experiments. SMZ collected the study individuals, performed the mate-choice experiments, and analysed all data. The manuscript was written by SMZ with contributions from JMS.

### Chapter 4: Opportunistic mate choice strategies in the orb-web spider *Argiope lobata*

JMS conceived and designed the experiments. SMZ performed the mating experiments, analysed all data and wrote the manuscript, with contributions from JMS.

### Chapter 5: Context- and state-dependent male mate choice in a sexually cannibalistic spider

JMS conceived and designed the experiments. SMZ collected the study individuals. Anna Gatz and Kristin Sauerland performed the first two mate-choice experiments (I & IIa). SMZ performed the last mate-choice experiment (IIb). The manuscript was written by JMS with contributions from SMZ.

### Chapter 6: Can males detect the strength of sperm competition and presence of genital plugs during mate choice?

JMS, Marie E. Herberstein (MEH) and SMZ conceived and designed the experiments. SMZ collected the study individuals with assistance from MEH. SMZ performed the mate-choice experiments, analysed all data and wrote the paper, with contribution from JMS und MEH.



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