

Sexual selection in sexually dimorphic dwarf spiders

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“Evolutionary biology aims to understand the diversity of life and the processes responsible for shaping it.” (Stearns & Hoekstra 2005, p. 1)

Summary

An individual's evolutionary fitness is determined by traits that evolved under natural and sexual selection. Natural selection supports traits that increase an individual's survival probability, whereas sexual selection favours traits that increase mating probability and mating success. All processes and traits involved before, during and after mating can be subject to sexual selection. Males can in general increase their evolutionary fitness by increasing number of mates, since each mating represents a chance to fertilize eggs. For females, on the other hand, one or few matings may be sufficient for fertilizing all of their eggs, resulting in pronounced female mate choice. Consequently, males often compete directly with each other for access to females including harmless displays (visual, vocal) or dangerous fights. In species in which females mate with more than one male, male-male competition continues after copulation in that sperm from different males compete over access to the eggs (sperm competition). Therefore, behaviour that reduces a male's risk of sperm competition in the first place will be selectively favoured. Adaptations that impede or reduce sperm competition comprise offensive mechanisms that reduce mating success of previous males, and defensive mechanisms that help to prevent females from remating.

The aim of this thesis was to investigate sexual selection processes and mechanisms in the closely related dwarf spiders *Oedothorax retusus* and *O. apicatus*. Dwarf spiders (Linyphiidae, Erigoninae) are especially suitable for sexual selection research as many of them exhibit sexual dimorphism, with males possessing modified prosomata. In those species that have been investigated in detail, and in which males possess a modified prosomal region, the modified structures are equipped with a glandular tissue that produces secretions, which the females contact and take up during courtship/copulation. The time of secretion release, and the time of potential refilling of the glandular reservoirs was analysed on an ultrastructural level in male *O. retusus* dwarf spiders. Males that only performed courtship but did not have physical contact with the females and males three days after mating had similar secretion amounts like males that never had contact to females. Immediately after mating, male glandular canals exhibited significantly less secretion compared with the other treatment groups. This strongly suggests that the main function of the secretions is gustatorial courtship and not the emission of volatile pheromones for mate attraction as previously assumed.

Mating decisions and reproductive success are influenced by secondary sexual traits that evolved under sexual selection. However, the nutritional status of the individual is also important for mate choice. Diet restriction during different developmental stages has been shown to influence mate choice, especially in females. Since spiders are regularly exposed to limited prey availability, adult feeding status can be considered an important component of spider mating behaviour. In order to

test for the effects of dietary restriction, females of the closely related dwarf spider species *O. retusus* and *O. apicatus* were subject to a short period of food shortage. The effects of low- (LD) vs. high-diet (HD) treatment on courtship, mating probability and behaviour, as well as reproduction were analysed. Female body weight (mg) served as a measure of fecundity. In both species, females from the LD treatment were less likely to mate. Copulation duration decreased in LD *O. retusus* females, but not in *O. apicatus*. Furthermore, LD females of both species produced lighter egg sacs containing fewer eggs, and started oviposition later than HD females. Obviously, short phases of diet restriction as adults have a high impact on copulation and reproduction in the two dwarf spider species.

Whenever females mate with more than one male, and sperm is stored prior to fertilization, males may suffer from sperm competition. Mating plugs that block the female genital openings after mating are a male strategy to avoid or reduce sperm competition and increase their reproductive success. Although mating plugs are supposed to occur in many species, their function and origin has hardly been investigated. *O. retusus* males are known to transfer amorphous material onto the female genitalia during mating. We investigated the location of plug production using x-ray microtomography (μ CT) as well as light and transmission electron microscopy (TEM). Furthermore, we asked whether males are limited in the production of the amorphous plug material in successive matings. The plug material is produced in a gland inside the male pedipalp and stored close to the blind end of the spermophore (the sperm storage compartment inside the male pedipalp). Consequently, the material must be released after sperm transfer to the female. Our study shows that the size of the first mating plug a male produced significantly influenced the size of subsequent plugs. Obviously, males do not possess unlimited amounts of plug material in a certain period, which may severely limit their ability to secure paternity through subsequent mating plugs.

Even though mating plugs seem to be an obvious means to secure paternity, their potential in securing paternity depends on their mechanical efficacy and persistence. Consequently, the influence of the size of the plug material (mating duration as a proxy) and the age of the mating plug (time interval between successive copulations) on its efficacy was investigated. Mating probabilities of subsequent males in the dwarf spider *O. retusus* were reduced, if the previous male placed a mating plug on /in the female genital opening. A high number (82%) of subsequent males tried to mate, but only 32.5% succeeded. Obviously, the mating plugs effectively prevent females from remating. Plug size and plug age significantly affected remating probability. Small and fresh plugs were least effective, whereas large plugs were highly effective independent of plug age. We were able to show that mating plugs in *O. retusus* are a powerful mechanical safeguard whose efficacy varies with plug size and age.

Genitalia in animals with internal fertilization are complex, species-specific, and underlie rapid evolution. In spiders, male and female genitalia are typically paired, and have to interact during mating, which results in an even higher complexity. Pedipalps (transformed pair of legs) in male spiders are used as secondary sperm transfer organs that are not directly connected to the gonads. As a result of the high complexity of male pedipalps, it has so far been taken for granted that pedipalps are side specific and cannot be used flexibly into either female copulatory opening. However, some cases of flexible pedipalp use have been documented. If versatile pedipalp use exists, this will be especially advantageous in species with mating plugs, since it allows males to circumvent a blocked (mated) female genital opening. Furthermore, males can compensate for a damaged pedipalp (moulting problems, predatory attack) by using the remaining functional pedipalp for both female genital openings. In the course of analysing mating plug efficacy, potential flexible pedipalp use was investigated in the dwarf spider *O. retusus*. Our findings demonstrate a flexible insertion mode in *O. retusus* - a dwarf spider with highly complex pedipalps but relatively simple female genitalia. This flexibility allows males to react to the mating history of the female by mating into the unused side, and thereby circumventing the highly effective mating plugs. Our findings corroborate sexual selection as the selective regime for the evolution of complex and diverse genitalia.

The results of this thesis show how complex sexual selection acts in the dwarf spiders *O. retusus* and *O. apicatus*. It shapes the evolution of male and female genitalia, affects mate choice (both pre- and postcopulatory), mating behaviour, and influences mating success and reproduction. All these factors and traits affect an individual's evolutionary fitness, and their interactions help to understand how sexual selection acts.

1 Introduction

1.1 Sexual selection and sexual conflict

An individual's evolutionary fitness is determined by different factors and traits that evolved under natural and sexual selection. Fitness can be defined as an individual's overall reproductive success that is composed of the survival-probability and the reproductive-probability (Kappeler 2012, p. 33). In order to be able to reproduce, an individual needs to survive until adulthood (Kappeler 2012, p. 33). Natural selection supports traits that increase an individual's survival probability, whereas sexual selection favours traits that increase mating probability and mating success (Krebs & Davies 1993, p. 183). Darwin (1859) described natural and sexual selection as two processes that can operate in the same direction, since individuals that are adapted optimally to certain circumstances produce the highest number of offspring, which adds to their evolutionary fitness. However, traits that are advantageous for sexual selection can be detrimental under natural selection. Such costly characters are mostly found in males and referred to as "secondary sexual characters" (Ridley 2004, p. 328). These traits are often conspicuous and sometimes deleterious, as they may reduce survival probability of the trait bearer (Clutton-Brock 2007). Sexual selection includes a variety of processes that all contribute to mating success/reproduction, such as time spent searching for mates, competition for food or mates, courtship, courtship feeding, mate choice, fecundity, or mate quality (Birkhead & Møller 1998, p. 56). All processes and traits involved before, during and after mating can be subject to sexual selection (Pélissier et al. 2014). Consequently, any interaction between the sexes that implicates fitness variations can influence sexual selection (Hall et al. 2008). In order to understand how these processes shape male and female traits and preferences, it is important to understand how they interact (Bonduriansky & Rowe 2003). Empirical studies mostly concentrate on mate choice of one sex, or competition among individuals of the other sex, which provides an incomplete view on the evolution of traits under sexual selection (Hunt et al. 2008). Sexual selection mechanisms can occur at the same time or sequentially, but irrespective of their chronology, their interactions help to understand how sexual selection works (Hunt et al. 2008).

Reproduction requires interactions between the sexes. However, in many cases the optima for males and females are not reached at the same time (Parker 2006), which can lead to sexual conflict. Males and females often have different evolutionary interests, and optimal trait values differ between the sexes (Arnqvist & Rowe 2005, p. 29, Arnqvist 2004, Chapman 2006, Wedell et al. 2006). Whereas monogamy should involve harmonic interactions between the sexes, sexual conflict will increase with multiple mating (Hosken et al. 2001). The resulting conflicts can intensify selective processes (Andersson & Simmons 2006), and have a high impact on mating, mating behaviour, and reproduction.

1.1.1 Intrasexual selection: competition for mates

Direct competition

Whenever sex ratios are unequal, and one sex is the limiting factor for the other, this can result in conflicting selective processes within one sex (Emlen & Oring 1977). Females often represent the limiting factor for males, as they are not constantly available for mating (Gwynne 1991). Consequently, males of many species compete with each other for access to females, or for breeding territories. Males can engage in direct competitions with other males that include harmless displays (visual, vocal) or dangerous fights. Traits that increase a male's competitive ability, and increase his potential reproductive rate, should be favoured by sexual selection (Clutton-Brock 2007). Females also compete with each other, mostly for resources (breeding territories, nutritive resources) that are important for breeding and rearing offspring (Clutton-Brock 2007, 2009). However, females also compete for access to mates, for example when sex ratios are female biased (Emlen & Oring 1977), or if they directly or indirectly benefit from mating (Clutton-Brock 2009). Female competition should be favoured by selection, whenever this results in increased fitness and mating success (Rosvall 2011).

Indirect competition: sperm competition

In polyandrous species in which sperm is stored within the female reproductive tract prior to fertilization, continues after copulation in that sperm from different males compete over access to the eggs (sperm competition) (Parker 1970, Birkhead & Møller 1998, Elgar 1998, Simmons 2001a). Males have a high evolutionary interest in fertilizing as many eggs as possible. Thus, how soon the female a male just mated with remates with another male strongly influences his reproductive success (Wedell et al. 2006). Therefore, behaviour that reduces a male's risk of sperm competition in the first place will be selectively favoured. Within the invertebrates, sperm competition and mechanisms to prevent sperm competition are especially widespread and diverse (Simmons 2001a). Adaptations that impede or reduce sperm competition comprise offensive mechanisms that reduce mating success of previous males, and defensive mechanisms that help to prevent females from remating (Parker 1970, 1998). Offensive mechanisms are destruction, removal, or displacement of rival sperm (reviewed in: Uhl et al. 2010). Defensive adaptations are mate guarding, reduction of female attractiveness (by means of antiaphrodisiacs), or reduction of female receptivity. A common defensive mechanism is the placement of mating plugs on or in the female genital openings (reviewed in: Uhl et al. 2010).

1.1.2 Intersexual selection: mate choice

Males and females usually have different reproductive optima, which mostly results in females choosing their mates according to quality indicators. Males can increase their evolutionary fitness by increasing number of mates, since each mating represents a chance to fertilize eggs (Arnqvist & Nilsson 2000, Gage et al. 2002). For females, on the other hand, one or few matings may be sufficient for fertilizing all of their eggs (Krebs & Davies 1993, pp. 176-177). Females invest a lot of energy into egg production and often into breeding of the offspring. The lower potential lifetime reproduction (limited egg numbers) in females compared with males, results in females being the limiting factor for males (Stearns & Hoekstra 2005, p. 248). The limiting sex (with lower reproductive potential) can be choosy, whilst the other sex (with higher reproductive potential) competes for access to mates (Stearns & Hoekstra 2005, p. 248). In many species, this results in pronounced mate choice (i.e. intersexual selection) for optimal mating partners (Krebs & Davies 1993, p. 186 ff., Andersson 1994).

Mate choice forces sexual selection on the other sex and accounts for impressive ornaments that can hardly be explained by natural selection (Kokko et al. 2003). Although mate choice occurs in both sexes, female choice of male traits is more obvious and females are considered choosier, since they invest more in their gametes (Tregenza & Wedell 2003). In most cases, females perform mate choice based on male behaviour, male traits, or distribution (Cotton et al. 2006). They can choose according to characters that signal direct or indirect benefits (Møller & Jennions 2001). Direct benefits can be increased fertility, higher fecundity, provision of paternal care, or courtship feeding (Møller & Jennions 2001, Wedell et al. 2006). Indirect benefits are genetic benefits, like increased offspring survival (genetically based viability or parasite resistance), or increased attractiveness of sons to females in the next generation (Møller & Jennions 2001). Different mechanisms aim at explaining the evolution of female choice: (1) Females may gain direct benefits (material benefits, nutrition, parental care) from mating with certain males (Andersson & Simmons 2006). (2) Female choice can be a result of the “runaway process” (or “sexy son theory”). This is a model of coevolution, first described by Fisher in 1915. He explained that females choose males with certain attractive characters that are genetically passed on to their sons in the next generation (Kappeler 2012, pp. 363-367). This is based on the assumption that an individual’s fitness does not only account for the number of offspring, which it produces, but also for the success of these offspring (Fisher 1915). When the female’s preference for a trait and the trait itself have a genetic basis, and are genetically linked, this results in a runaway process: The female’s preference is intensified and at the same time, the preferred ornament/character is increased (Ridley 2004, p. 330). (3) Another theory is the “handicap principle”, proposed by Zahavi in 1975. The occurrence of mate choice can be explained according to indicators of broad genetic quality (“good genes” or “handicap mechanisms”)

(Andersson & Simmons 2006). The “handicap principle” describes how costly male traits can be advantageous for females (Smith 1976, Ridley 2004, pp. 331-332). Producing conspicuous traits is costly; consequently, only high quality males can afford such traits (Kappeler 2012, p. 367). These characters are honest and reliable indicators of male quality, as they reflect a male’s ability to withstand infections and survive despite such deleterious traits (Ridley 2004, p. 332). There is growing interest in the idea that (4) genetic compatibility is a force that drives mate choice (Tregenza & Wedell 2003). The underlying idea is that females can increase their own and their offspring’s fitness by choosing a mate according to highly compatible alleles (Puurtinen et al. 2009). Mechanisms one to four can occur at the same time and are compatible, meaning that the evolution of preferences for certain traits is a “multiple-causation problem” (Andersson & Simmons 2006).

Cryptic female choice

Sexual selection on male traits continues after copulation, via sperm competition and cryptic female choice (Birkhead & Pizzari 2002, Aisenberg & Eberhard 2009). Cryptic female choice is the postcopulatory equivalent of female mate choice. Female preferences for sperm of certain males cannot be observed directly, but strongly influence a male’s paternity share (Andersson & Simmons 2006). Prerequisites for cryptic female choice are multiple female mating, differential female responses to male traits that affect a male’s chances to sire offspring, and correlations between female responses and male traits (Aisenberg & Eberhard 2009). Even if a male is accepted as a mating partner, this does not necessarily result in paternity success (Eberhard 1996). Many mechanisms are known by which females can perform cryptic female choice (Eberhard 1996, Aisenberg & Eberhard 2009). The sperm that the females store within their reproductive tract represent their cryptic choice, however, the amount of stored sperm can also reflect male responses to the female (Andersson & Simmons 2006). As with precopulatory mate choice, it is not always clear whether we observe male or female mate choice (Andersson & Simmons 2006).

1.1.3 Secondary sexual characters

The highest diversity between related species is found in traits that differ between the sexes (Arnqvist 2006). Secondary sexual characters are features that already concerned Darwin (1871). They differ between males and females, are developed as an individual reaches maturity, are important for reproduction, but are not directly connected to the primary sexual organs (Clutton-Brock 2007, 2009). An increasing number of studies investigate conspicuous sexual dimorphism of many different species (Andersson 1994, Clutton-Brock 2007). The analysed traits show high variance between closely related species and taxa, and are subject to rapid evolution (Arnqvist 2006). The driving forces in the evolution of secondary sexual characters seem to be mating preferences of one

sex, and/or intrasexual competition for mating partners (Clutton-Brock 2007, 2009). Secondary sexual characters are found in both sexes, and are shaped by intra- and/or intersexual selection to attract mates (Clutton-Brock 2009). Some secondary sex characters like greater body size, weapons (antlers, horns, spines, teeth, etc.), or ornamentation are rather obvious. Sexual selection has been accepted as the underlying force for the evolution of secondary characters such as weapons (Andersson 1982). However, advantageous characters that increase mating probability or reproductive success can also be less conspicuous, and it is less clear what the underlying evolutionary forces are. Variability in mate preferences for male secondary sexual traits can put a high selection pressure on individuals to present their qualities as mating partners in order to be favoured (Clutton-Brock 2007).

1.2 Mating and mating strategies

Mating systems are very diverse, and all aim at securing reproduction and maximizing an individual's evolutionary fitness (Kappeler 2012, pp. 392, 543 ff.). Categorizations of mating systems have been proposed several times (Emlen & Oring 1977). They are usually defined by the number of mating partners one sex achieves (Emlen & Oring 1977). Sexual reproduction with two separate sexes is most common in the animal kingdom, and has been investigated in detail in many species (Kappeler 2012, p. 246). Polyandry is a widespread mating system, since females often benefit from mating with more than one male (Arnqvist & Nilsson 2000). Polyandrous matings can be advantageous for females, if they increase fecundity (Gwynne 2008), fertility, or if females receive extra nutrition through nuptial gifts (Vahed 1998, Gwynne 2008), sexual cannibalism (Elgar 1998), or fluids that are transferred with the sperm. Furthermore, females can increase their offspring's genetic diversity by mating with different males (Arnqvist & Nilsson 2000). Overall, females that mate multiply have a higher lifetime reproductive success that outweighs potential negative effects of remating, like reduced lifetime (Arnqvist & Nilsson 2000, Wedell et al. 2006). In most species an optimal intermediate female mating rate is observed (Arnqvist & Nilsson 2000).

1.2.1 Courtship feeding and nuptial gifts

Transfer of nutritional substances from the male to the female during courtship and/or copulation is called courtship feeding or nuptial feeding (Vahed 1998). Even though conflict exists between males and females over mating rates and/or duration, matings that include transfer of nuptial gifts have been proposed an example of cooperation between the sexes (Vahed 2007). Nuptial gifts are very common in arthropods, and can consist of prey items that the male collected, male body parts (or the complete male), glandular or salivary secretions, or substances within the ejaculate or the spermatophore (Vahed 1998, Gwynne 2008). The gifts can be beneficial to the female and have high nutritional values (Gwynne 2008). They can function as paternal investment, by increasing the gift-giving male's paternity success. Many studies on insects suggest that different forms of courtship/nuptial feeding serve as a mating effort (Vahed 1998). This is the case if the substances attract females, support mating, and/or maximize sperm transfer (Vahed 1998, Gwynne 2008). However, nuptial gifts can also provide little direct benefits, or even be detrimental to the female. They can function as a "sensory trap": by taking advantage of the female's gustatory responses, the male is able to entice the female to mate, to mate longer, or to transfer higher amounts of sperm than is in the interest of the female (Vahed 2007). Consequently, some nuptial gifts can entice the female to mate and result in numbers of copulations that exceed the female's optimal mating rate (Vahed 2007).

1.2.2 *Mating plugs*

Whenever females mate with more than one male, and sperm is stored prior to fertilization, males may suffer from sperm competition. In order to prevent females from mating multiply, males evolved different adaptations, for example guarding of the female after mating, transfer of substances that decrease female attractiveness (antiaphrodisiacs), or the production of substances that block the female genital openings (i. e. mating plugs) (Baer et al. 2000, for more examples see: Arnqvist & Rowe 2005, pp. 108-109).

Mating plugs evolved in many species of different taxa (Birkhead & Møller 1998, Uhl & Busch 2009, Uhl et al. 2010). They help the male to reduce sperm competition risk and reduce the female's potential for cryptic choice (Birkhead & Møller 1998, Uhl & Busch 2009). By applying mating plugs and physically blocking the female genital tract males directly influence female mating rates (Fromhage 2011, Schneider & Andrade 2011, p. 234). Mating plugs can consist of different materials: amorphous substances, male genital fragments or the complete male (Uhl et al. 2010, Herberstein et al. 2012). However, what determines mating plug efficacy needs to be investigated and tested (Uhl et al. 2010). For some species, plug efficacy has been shown to depend on persistence of the plug material and/or the size of the plug (Uhl & Busch 2009, Uhl et al. 2010, Herberstein et al. 2012). In many supposed mating plugs, their origin and function are unknown, and it is not known how long lasting the plugs are (Uhl et al. 2010). To fully understand how mating plugs evolved, all aspects of intersexual interactions (morphological, behavioural, biochemical) need to be explored (Uhl et al. 2010).

1.3 Spiders as study organisms

Model systems suitable for sexual selection research should be characterized by a sexual dimorphism (morphological, behavioural), since these mostly evolved under sexual selection (Andersson & Simmons 2006). Sexual selection research requires experimental treatments and analysis; therefore, model systems should have small body sizes and short generation times, in order to be able to have many replicates, or to test effects in next generations (Andersson & Simmons 2006).

Spiders (Araneae) are a very diverse group with currently 45144 species from 3935 genera (Platnick 2015). They have a worldwide distribution in almost all environments. The great spider diversity is not only a result of the high species numbers, but also arises from the unique and complex behaviours many spider species exhibit. Spiders bear several characters that make them very suitable for sexual selection research (Eberhard 2004). Males possess secondary sperm transfer organs that are not directly connected to the gonads and can be relatively easily manipulated. Male and female genitalia are paired, which leads to the advantage, that an individual can serve as the experimental and control animal at the same time (Eberhard 2004). Taxonomic studies have revealed ample information about the morphology of male and female genitalia, which makes it possible to analyse evolution and divergence of genital structures, as well as the morphological interactions between the sexes (Eberhard 2004). Furthermore, sperm is transferred in an immobile state, which allows the investigation of cryptic female choice and sperm competition within the female reproductive tract (Eberhard 2004).

1.3.1 Spider phylogeny

Spiders (Araneae) belong to the Arachnids, and based on characteristics like cheliceral fangs with venom glands, silk glands, and modified legs (male pedipalps that serve for sperm transfer), spiders are a monophyletic group (Coddington & Levi 1991). According to their genital structures, spiders are divided into haplogyne or entelegyne spiders (Foelix 2011, pp. 5-6). Haplogyne spider females possess unpaired genital openings that serve for both - copulation and oviposition. Entelegyne spider females have three genital openings: two copulatory openings, and a third opening for oviposition. Consequently, copulation ducts in entelegyne females are separated from the fertilization ducts, unlike in haplogyne females (Herberstein & Wignall 2011, pp. 4, 11).

1.3.2 Spider morphology

The spider body consists of two main regions: the prosoma, and the opisthosoma that are connected via the pedicel (Herberstein & Wignall 2011, p. 4). The prosoma is used for feeding (chelicerae and venom glands), locomotion (4 pairs of legs), and contains the nervous system. The

first pair of body appendages following the chelicerae are the pedipalps. They are primarily used for inspecting prey or the ground (Foelix 2011, p. 24). In males, however, they serve as secondary sperm transfer organs. In order to charge his pedipalps with sperm, the male builds a small horizontal sperm web, releases a sperm drop from the testes to the web, and takes up the sperm into a storage compartment (spermophore) inside the genital bulb (the terminal part of the pedipalp) (Foelix 2011, pp. 24, 232-233, Herberstein & Wignall 2011, p. 10). The opisthosoma is used for respiration, digestion, excretion, reproduction, and bears the spinnerets for silk production (Foelix 2011, pp. 37-39). Furthermore, male and female genital openings are situated on the ventral side of the opisthosoma.

1.3.3 Dwarf spiders (*Araneae, Linyphiidae, Erigoninae*)

Dwarf spiders represent the largest subfamily within the Linyphiidae (Hormiga 2000). Dwarf spiders make up about 25 % of the complete spider fauna of northern Europe (Hormiga 2000). They comprise species that reach a mean body size of 2 mm (range 1-6 mm) (Hormiga 2000). Some male dwarf spiders are characterized by diverse modified prosomata, with lobes, humps, grooves, pits, and hairs (Fig. 1). Behavioural observations revealed that in some species, the females contact the modified regions either with their mouth opening, or with their chelicerae, and feed on secretions that the males offer from these structures (Bristowe 1931, Schlegelmilch 1974, Uhl & Maelfait 2008, Kunz et al. 2012).

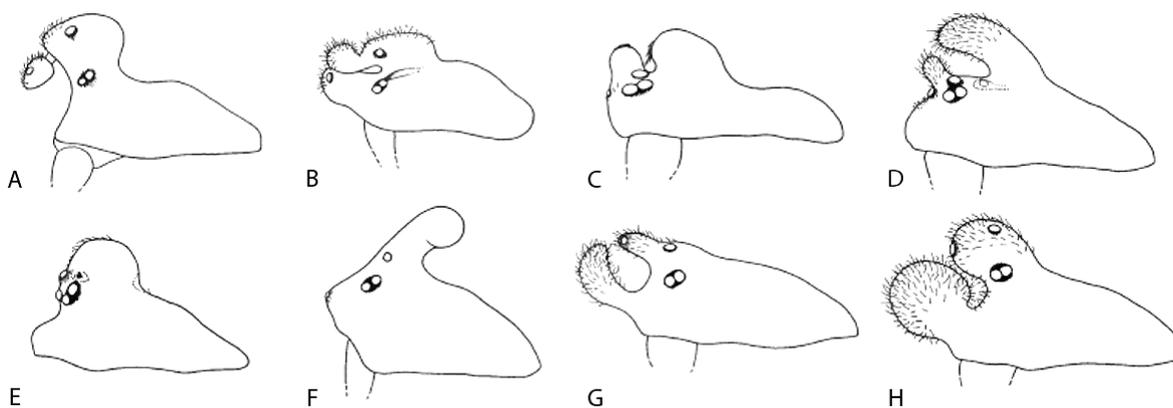


Fig. 1 — Lateral view on prosomata of male dwarfs spiders (Erigoninae) with prosomal modifications (modified after Hormiga 2000). (A) *Dactylopiastes mirabilis* (B) *Savignia birostrum* (C) *Bursellia cameroonensis* (D) *Scotinotylus majesticus* (E) *Dismodicus bifrons* (F) *Minicia kirghizica* (G) *Coreogonal bicornis* (H) *Coreogonal petulcus*.

1.3.4 Spider mating behaviour

In order to find or attract a mating partner, spiders use different cues such as mechanical (silk lines), or chemical signals (pheromones) (Schneider & Andrade 2011, p. 216 ff.). When potential mating partners meet, most spiders exhibit species-specific precopulatory courtship behaviours (sequences consisting of visual and tactile signals) (Schneider & Andrade 2011, p. 220). Between and within species and taxa, courtship varies not only in behaviours and sequences, but also in duration (Schneider & Andrade 2011, p. 223). Differences in courtship duration may be the result of evolutionary processes that balance costs and benefits for prolonged courtship between taxa (Schneider & Andrade 2011, p. 223). Overall, courtship highly determines mating success in many spider species (Schneider & Andrade 2011, p. 220). After courtship, male mating success depends on the female's mate choice decision and on presence of competitors of the same sex. Often, females respond differently to courting males, which suggests that the females choose amongst males, according to behavioural or morphological male traits that represent some advantage for the female (Schneider & Andrade 2011, p. 225).

Female mate choice decisions also seem to be influenced by the female's previous experiences, which should be included in mate choice analyses (Schneider & Andrade 2011, p. 227). Whenever females are likely to attract more than one male at a time, the rival males will compete with each other for access to the female (Schneider & Andrade 2011, p. 229). In spiders, cryptic processes of choice and competition are very common, and many species are polyandrous. Consequently, mating success of a certain male is not only determined by courtship success and subsequent copulation, but also by sperm competition and cryptic female choice within the female reproductive tract (Schneider & Andrade 2011, p. 233). In order to reduce female mating rates, males apply mating plugs (Schneider & Andrade 2011, p. 234). This is especially widespread in entelegyne spiders. Entelegyne spider females have three genital openings: two are used for copulation, and one for oviposition (Uhl et al. 2010). This allows the male to obstruct the copulatory ducts with mating plugs without impeding oviposition (Uhl et al. 2010, Schneider & Andrade 2011, p. 234).

1.3.5 Courtship and mating in *Oedothorax* species

Courtship and mating includes a series of different behaviours that are alike in the two investigated *Oedothorax* species: *Oedothorax retusus* and *O. apicatus*. As soon as the male perceives the presence of a female, he starts his typical courtship behaviour (Kunz et al. 2012). This includes vibrating with his opisthosoma, trembling with his first pair of legs, plucking on the female's web, and cleaning his pedipalps while he approaches the female. Females often show their interest by turning towards the male, and otherwise remaining motionless. The mating position is antiparallel with the ventral sides turned upwards (Fig. 2C) (von Helversen 1976). In mating position, the female contacts

the modified male prosomal region (Fig.2C, right arrowhead), and often releases saliva onto the structures, and reingests it (Kunz et al. 2012, 2013). Copulation starts as soon as the male couple's one of his pedipalps to the female copulatory opening, and the membranous part of his sperm transfer organ (the hematodocha, Fig. 2C, left arrowhead) inflates (Kunz et al. 2012). At the end of an insertion, the male applies an amorphous mating plug to the female copulatory opening that prevents the female from remating (Uhl & Busch 2009). Copulation can consist of one insertion (with one pedipalp) or two sequential insertions (with both pedipalps), resulting in one or two mating plugs.

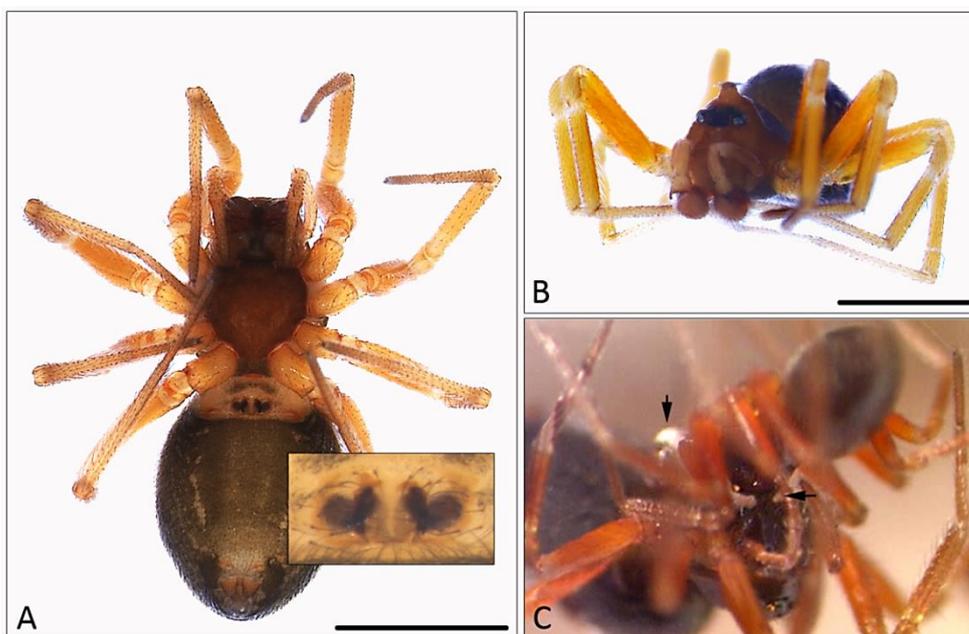


Fig. 2 — (A) Ventral view of an *Oedothorax apicatus* female. Inserted figure: female epigyne (genital region). (B) Anterior view of an *O. apicatus* male. Characteristic is the males modified prosomal region (hairy protuberance). (C) Male and female *O. apicatus* in antiparallel mating position (upside down in the web). Female on the lower left, and male on the upper right. The left arrowhead marks the expanded hematodocha; the right arrowhead marks the contact-area between male protuberance and female moth opening. Scale bars: 1mm.

Some dwarf spiders are characterized by a sexual dimorphism with males possessing peculiar prosomal (“cephalic”) modifications (Hormiga 2000, Fig. 1). *O. apicatus* males feature a prominent turret behind the eye region (Fig. 2B), and lateral sulci with pits (Michalik & Uhl 2011). *O. retusus* males have a steep hump behind the ocular region (Fig. 3C, circle), and two deep invaginations (sulci with pits, Fig. 3C, arrowhead) on both sides of the prosoma (Michalik & Uhl 2011). The modified male

prosomal regions of both *O. retusus* and *O. apicatus* are equipped with massive glandular tissue (Michalik & Uhl 2011). The secretions that are produced from the glandular tissue have been shown to affect mating probabilities and reproduction in *O. retusus* (Kunz et al. 2012).

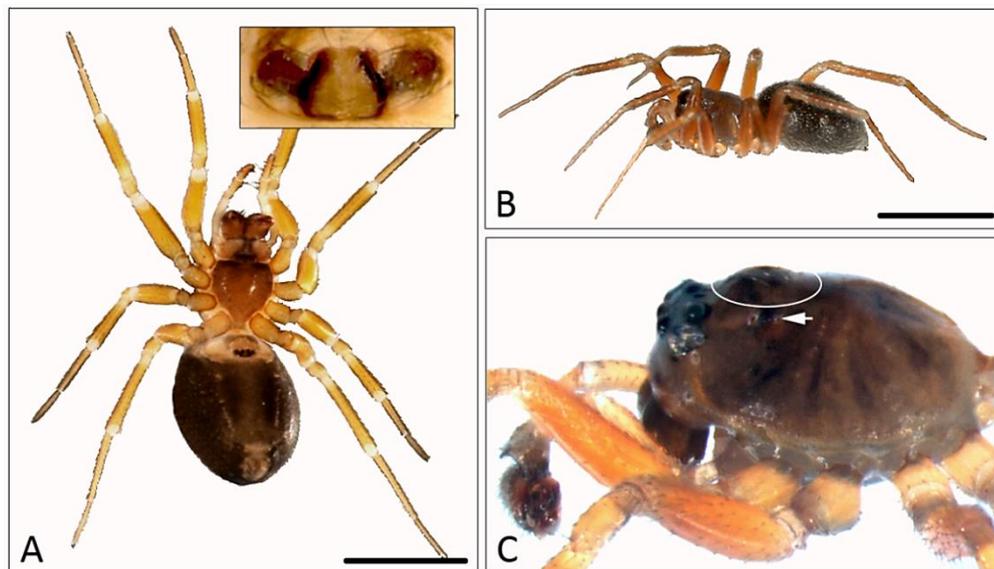


Fig. 3 — (A) Ventral view of an *Oedothorax retusus* female. Inserted figure: female epigyne (genital region). (B) Lateral view of an *O. retusus* male. Characteristic is the modified prosomal region (hump with lateral sulci and pits). (C) Enlarged view of the prosoma of an *O. retusus* male. The circle marks the hump-area, and the arrowhead marks the left sulcus with pit. Scale bars: 1mm.

1.3.6 Study specimen: Origin, rearing and maintenance

We used two species of the European dwarf spider genus *Oedothorax* (Linyphiidae, Erigoninae) as focal species. *Oedothorax retusus* is very frequent on salt marshes and riverbanks, whereas *Oedothorax apicatus* mostly inhabits cultivated, agricultural areas (Kunz et al. 2012, Alderweireldt & De Keer 1988). The body length of adult females is between 2.5 – 3mm (*O. apicatus*: Fig. 2A, *O. retusus*: Fig. 3A), adult males reach about 2 mm (*O. apicatus*: Fig. 2B, *O. retusus*: Fig. 3B). As most entelegyne spiders, female *O. retusus* and *O. apicatus* possess paired genitalia, with two separate copulatory openings and two ducts that lead to a spermatheca each. As in all spiders, males use their complex paired pedipalps as secondary copulatory organs (Foelix 2011, p. 24).

The specimen that were used for the studies were raised from egg sacs of wild caught females. Female *O. retusus* were collected from the banks of the River Rhine (Fig. 4), near Bonn, Germany in spring (~April) and late summer/early autumn (~September) of the years 2009-2012. Female *O. apicatus* were collected from an agricultural field (Fig. 5) near Bonn Beuel, Germany also in spring

(~April) and late summer/early autumn (~September) of the years 2011-2012. Females were kept separately in 25 ml plastic containers under controlled conditions (23/17 °C (day/night), 70% humidity) (for details see: Kunz et al. 2012). After oviposition, egg sacs were kept separately, and as soon as spiderlings hatched from the egg sac, they were fed ad libitum on springtails (*Sinella curviseta*) (Kunz et al. 2012). In general, males and females were fed with four to six fruit flies per week (*Drosophila melanogaster*) (Kunz et al. 2012).

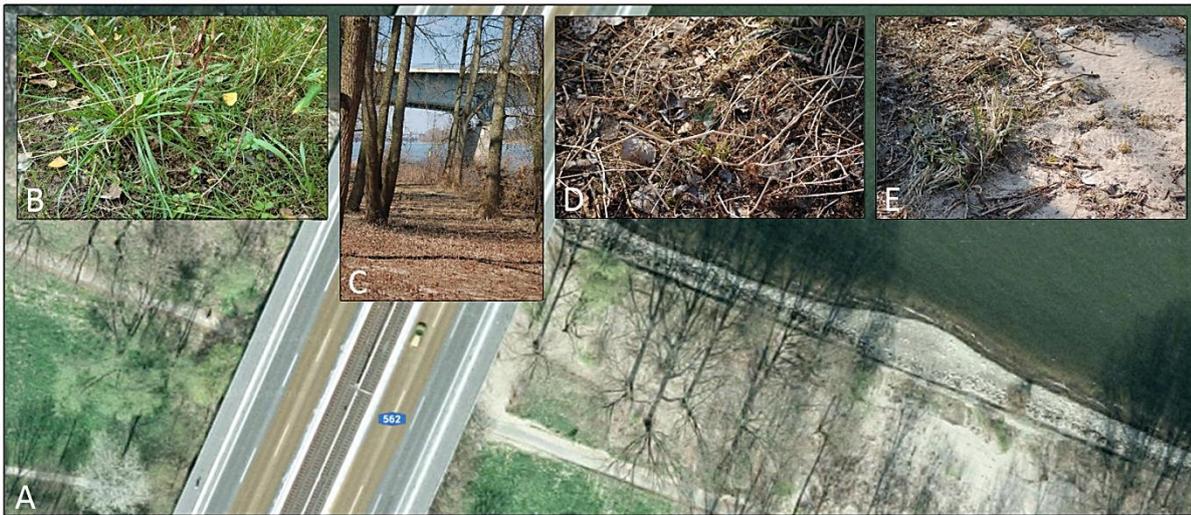


Fig. 4 — Collecting site of adult female *O. retusus*. (A) Aerial view of the collection site on the banks of the river Rhine, near Bonn, Germany (50°43'00.26"N, 7°08'25.11"E – 50°42'57.40"N, 7°08'36.70"E) (© Google earth). (B) View on the locality during spring. (C)-(E) Collecting site during autumn.



Fig. 5 — Collecting site of adult female *O. apicatus*. (A) Aerial view of the agricultural field in the vicinity of Bonn Beuel, Germany (50°45'19.16"N, 7°09'11.11"E - 50°45'17.76"N, 7°09'07.20"E) (© Google earth). (B)-(C) Detailed view on the habitat.

1.4 Study aims

Dwarf spiders (Linyphiidae, Erigoninae) are especially suitable for sexual selection research as many of them are characterized by a sexual dimorphism, with males possessing modified prosomata (Hormiga 2000, Michalik & Uhl 2011). The modifications play an important role during courtship and/or copulation in all species investigated so far (Bristowe 1931, Schlegelmilch 1974, Uhl & Maelfait 2008, Kunz et al. 2012). *O. retusus* males release secretions from their glandular tissue that affect mating probability and reproduction (Kunz et al. 2012). Furthermore, they produce amorphous mating plugs, which they apply to the female copulatory opening at the end of copulation, thereby affecting female remating probabilities (Uhl & Busch 2009). The aim of this thesis was to investigate sexual selection processes and mechanisms in the dwarf spiders *O. retusus* and *O. apicatus*, that are considered sister species (Lopardo & Uhl 2014).

1.4.1 Timing of prosomal secretion release

The aim of this study was to analyse the time of prosomal (“cephalic”) secretion release, and the time of potential refilling of the glandular reservoirs on an ultrastructural level in *O. retusus* males. Glandular tissue was compared between a) inexperienced males (that never had contact to females), b) males that performed courtship (but had no physical contact to females), c) males immediately after copulation, and d) males three days after mating. We analysed whether the treatment groups differed in the filling state of the conducting canals and reservoir regions (receiving canals) of the glandular tissue. Males that only performed courtship but did not have physical contact with the females, as well as males three days after mating, had secretion amounts similar to males that never had contact to females. Immediately after mating, male glandular canals exhibited significantly less secretion compared with the other treatment groups. This strongly suggests that the main function of the secretions is gustatorial courtship and not the emission of volatile pheromones for mate attraction as previously assumed.

For details, see manuscript “Kunz, K., Michalik, P. & Uhl, G. (2013). Cephalic secretion release in the male dwarf spider *Oedothorax retusus* (Linyphiidae: Erigoninae): An ultrastructural analysis. *Arthropod Structure & Development* 42, 477-482” (p. 25).

1.4.2 Effects of nutrition on mating decisions and reproductive output

Mating decisions and reproductive success are influenced by secondary sexual traits that evolved under sexual selection, but can also be affected by the nutritional status of the individual. The number and quality of potential mates affects optimal mate choice (Andersson & Iwasa 1996). Further, mate choice is not consistent during a lifetime (e.g. Brown & Kuns 2000), and depends on an individual’s own and the potential mates condition (Cotton et al. 2006, Holveck & Riebel 2010). Diet

restriction during different developmental stages has been shown to influence mate choice, especially in females. In order to understand if and how short periods of food limitation as adults influence male and female mating decisions, we restricted female food quantities. Since spiders are regularly exposed to limited prey availability, we consider adult feeding status an important component of mating behaviour that is likely to shape mate choice. In order to test this we conducted experiments in which females of the closely related dwarf spider species *O. retusus* and *O. apicatus* were subject to a short period of food shortage. We investigated the effect of low- vs. high-diet treatment on courtship, mating probability and behaviour, as well as reproduction. Female body weight (mg) at the day of the experiment serves as a measure of fecundity (Uhl et al. 2004). In both species, females from the low-diet treatment were less likely to mate. Copulation duration decreased in low-diet *O. retusus* females, but not in *O. apicatus*. Furthermore, low-diet females of both species produced lighter egg sacs containing fewer eggs, and started oviposition later than high-diet females. Obviously, short phases of diet restriction as adults have a high impact on copulation and reproduction in the two dwarf spider species.

For details, see manuscript “Kunz, K. & Uhl, G. (submitted). Short-term nutritional limitation effects mating behaviour and reproductive output in dwarf spiders. Submitted to *Ethology*” (p. 33).

1.4.3 Mating plugs: plug efficacy, plug production, and limitations

Mating plugs that block the female genital openings after mating are a male strategy to increase their reproductive success and avoid or reduce sperm competition. In many supposed mating plugs, their exact function and their origin remains to be investigated. *O. retusus* males produce mating plugs that secure paternity and prevent females from remating (Uhl & Busch 2009). We investigated the location of plug production using x-ray microtomography (μ CT) as well as light and transmission electron microscopy (TEM). Furthermore, we asked whether males are limited in the production of the amorphous plug material in successive matings. We found that the plug material originates from a gland inside the male pedipalp. The plug material is produced in a gland inside the male pedipalp and stored close to the blind end of the spermophore (the sperm storage compartment inside the male pedipalp). Consequently, the material must be released after sperm transfer to the female. Our study shows that the size of the first mating plug a male produced significantly influenced the size of subsequent plugs. Obviously, males do not possess unlimited amounts of plug material in a certain period, which may severely limit their ability to secure paternity through subsequent mating plugs.

For details, see manuscript “Uhl, G., Kunz, K., Vöcking, O. & Lipke, E. (2014). A spider mating plug: origin and constraints of production. Published in *Biological Journal of the Linnean Society* 113, 345-354” (p. 49).

Even though mating plugs seem to be an obvious means to secure paternity, their potential in securing paternity depends on their mechanical efficacy and persistence. We investigated the influence of the size of the plug material (mating duration as a proxy) and age of the mating plug (time interval between successive copulations) on its efficacy. We analysed mating probabilities of subsequent males in *O. retusus*. A high number (82%) of subsequent males tried to mate, but only 32.5% succeeded. Obviously, the mating plugs are highly effective in preventing females from remating. Plug size and plug age significantly affected remating probability. Small and fresh plugs were least effective, whereas large plugs were highly effective independent of plug age. Those males that were able to copulate despite a mating plug did not necessarily succeed in transferring sperm, since sperm masses were found on the plugged female genital area. We were able to show that mating plugs in *O. retusus* are a powerful mechanical safeguard whose efficacy varies with plug size and age (suggesting that hardening of the material is required).

For details, see manuscript “Kunz, K., Witthuhn, M. & Uhl, G. (2014). Do the size and age of mating plugs alter their efficacy in protecting paternity? Published in *Behavioral Ecology and Sociobiology* 68, 1321-1328” (p. 61).

1.4.4 Versatile pedipalp use despite complex genitalia

Genitalia in animals with internal fertilization are very complex and evolve rapidly. Consequently, male and female genitalia are highly species specific. In spiders, male and female genitalia are typically paired, and have to interact during mating, which results in an even higher complexity. Pedipalps in male spiders are used as secondary sperm transfer organs and are not directly connected to the gonads. During copulation, they have to interact with and couple to female outer and inner genital structures. Due to the high complexity of male pedipalps, it was taken for granted, that pedipalps are side specific and cannot be coupled to either female genital openings (von Helversen 1976). However, some cases of versatile pedipalp use have been documented, but only from species with rather simple pedipalps (e.g. Costa et al. 2000, Eberhard & Huber 1998, Fromhage & Schneider 2005). If versatile pedipalp use exists, this will be especially advantageous in species with mating plugs, since it allows males to react flexible to a blocked (mated) female genital opening. Further, males can compensate for a damaged pedipalp (moulting problems, predatory attack) by using the remaining functional pedipalp for both female genital openings. In the course of analysing mating plug efficacy, we investigated potential flexible pedipalp use in *O. retusus*. Our findings demonstrate a flexible insertion mode in *O. retusus* - a dwarf spider with highly complex pedipalps but relatively simple female genitalia. This flexibility allows males of this species to react to the mating history of the female by mating into the unused side, and thereby circumventing the highly

effective mating plugs. Our findings corroborate sexual selection as the selective regime for the evolution of complex and diverse genitalia.

For details, see manuscript “Kunz, K., Witthuhn, M. & Uhl, G. (in revision). Paired and complex copulatory organs: do they really impede flexible use? In revision: *Journal of Zoology*” (p. 71).

**Cephalic secretion release in the male dwarf spider *Oedothorax retusus*
(Linyphiidae: Erigoninae): An ultrastructural analysis**

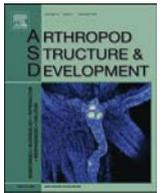
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Cephalic secretion release in the male dwarf spider *Oedothorax retusus* (Linyphiidae: Erigoninae): An ultrastructural analysis



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ABSTRACT

Secondary sexual traits in males can extend to glandular structures that play a role during courtship and mating. In dwarf spiders (Linyphiidae, Erigoninae), glandular secondary sexual traits are particularly common. Males are characterized by cephalic modifications which produce secretions that females contact with their mouthparts during courtship and/or copulation. We used the dwarf spider *Oedothorax retusus* as a model species to investigate if and when the contents of the glands are released during a mating sequence and if so, if the gland reservoirs are refilled after mating. To this aim, we quantitatively compared the glandular tissue on the ultrastructural level between a) inexperienced males, b) males that performed courtship, c) males immediately after copulation, and d) males three days after mating. We assessed whether the treatment groups differed in the filling state of the conducting canals and receiving canals (reservoir regions) of the glandular units. Our study shows that courting males as well as males three days after mating did not differ significantly from control (inexperienced) males in the presence of secretions. However, males exhibited significantly less secretion immediately after mating. This strongly suggests that the main function of the secretions is gustatorial courtship and not the emission of volatile pheromones for mate attraction as was previously assumed.

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1. Introduction

Secondary sexual traits that increase mating success of the male are known to be favored by sexual selection. Besides colorful and costly ornaments or weapons secondary sexual traits can also consist of glandular products that may entice the female to mate and/or may reveal the condition of the male (Vahed, 1998, 2007). Such glandular products entail external glandular secretions, salivary secretions, spermatophores or substances in the ejaculate (Vahed, 1998). External glandular secretions have evolved many times independently and can be found in beetles, zoraptera, crickets, gryllids, cockroaches (Vahed, 1998) and arachnids (Martens, 1973; Martens and Schawaller, 1977; Robinson, 1982; Juberthie-Jupeau et al., 1990; Huber, 1997, 2005; Vanacker et al., 2003; Maes et al., 2004; Uhl and Maelfait, 2008; Michalik and Uhl, 2011; Kunz et al., 2012). The secretions can be produced in almost any part of the male's body, including the head, the neck, the thorax, the forewings, and the legs (Vahed, 1998). These secretions are considered to

release volatile substances (pheromones) or produce substances that are taken up by the female during the mating sequence which is termed gustatorial courtship (Lopez, 1983; Andersson, 1994; Huber, 1997; Vahed, 1998; Bilde et al., 2007; Gwynne, 2008).

Males of dwarf spiders (Erigoninae) often have species-specific structures in or behind the ocular region of the prosoma. These secondary sexual traits may appear as knobs, turrets, grooves or humps (Wiehle, 1960; Hormiga, 2000; Arnedo et al., 2009). The structures are connected to exocrine glands in all species that have been investigated to date (Schaible et al., 1986; Schaible and Gack, 1987; Vanacker et al., 2003; Michalik and Uhl, 2011). The secretions have not yet been analyzed biochemically, but they are considered to function as volatile pheromones and/or consist of nutrients; they may also contain substances that influence cryptic female choice (Lopez, 1983; Eberhard, 2004; Huber, 2005). The available data on a handful of species show that the females contact the structures and probably ingest the glandular products during the mating sequence (Bristowe, 1931; Schlegelmilch, 1974; Vanacker et al., 2003; Maes et al., 2004; Kunz et al., 2012).

In our focal species *Oedothorax retusus*, males possess two characteristic cephalic structures that are connected to glandular tissue: a hump behind the ocular region and two lateral sulci with pits below the hump (de Causmaecker, 2004; Michalik and Uhl, 2011; Kunz

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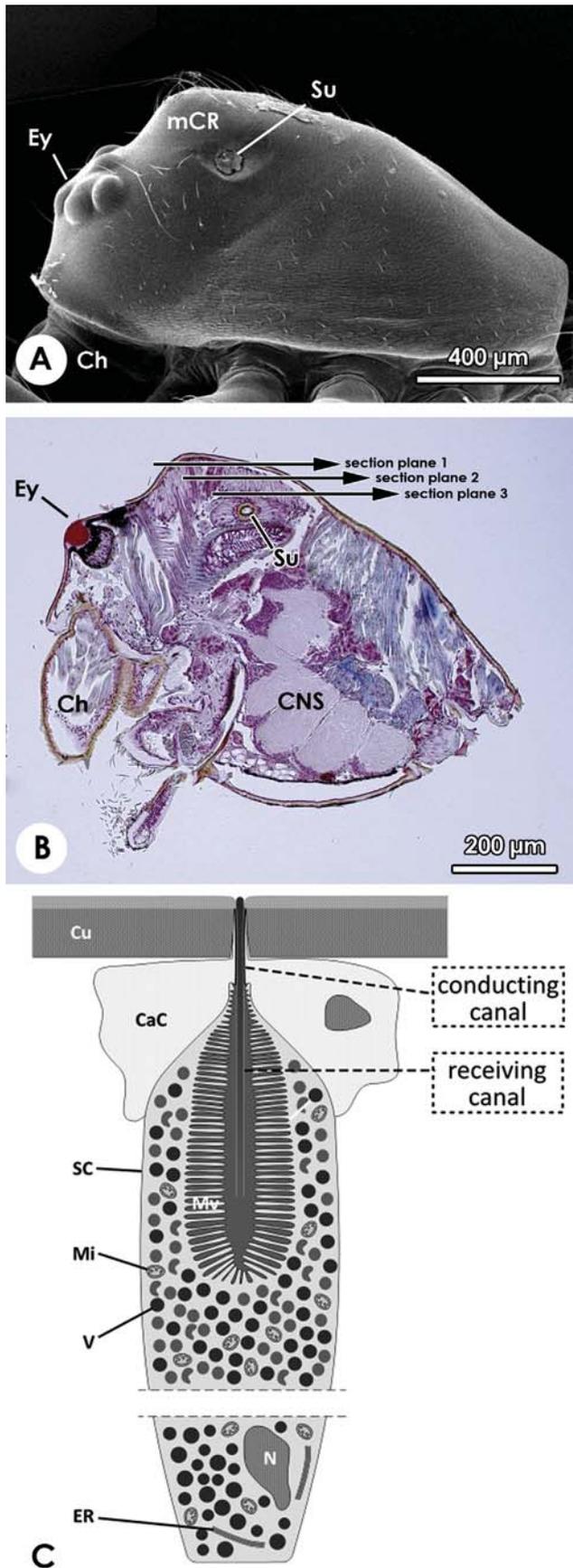


Fig. 1. (A) SEM-photograph showing a lateral view on the prosoma of an *Oedothorax retusus* male. (B) Histological sagittal section of the prosoma of an *O. retusus* male, with

et al., 2012). The glandular tissue consists of two gland types that are composed of vesicle-containing secretory cells and canal cells. The glandular products are released via conducting canals (cuticular canals) that extend into the receiving canals within the reservoir region of the secretory cells (Michalik and Uhl, 2011). During copulation female *O. retusus* contact the male cephalic structures while being in an antiparallel mating position (Kunz et al., 2012). Females can be observed to salivate onto the male cephalic hump and presumably ingest the liquefied male secretion (Kunz et al., 2012).

Although the behavioral observations on *O. retusus* strongly suggest that the secretions do not serve as volatile pheromones for mate attraction but are released during copulation while the female contacts the structure, it remains to be investigated if and when the glandular tissue releases its secretions. Our study provides the first quantitative data on the timing of the release of the glandular products. In addition, we investigated whether the glandular ducts and reservoirs are refilled after mating.

2. Materials and methods

2.1. Collection and rearing of dwarf spiders

O. retusus (Westring, 1851) is a common European dwarf spider species (Linyphiidae: Erigoninae) that occurs in wet habitats such as salt marshes and river banks (Alderweireldt and De Keer, 1988). Adult females were collected for egg laying from the banks of the river Rhine south of Bonn, Germany (50°43'00.26"N, 7°08'25.11"E–50°42'57.40"N, 7°08'36.70"E) in March/April and August/September 2010–2012. Specimens were kept individually in 25 ml plastic containers under controlled conditions (70% humidity, 20 °C/23 °C, 12:12 h photoperiod). Spiderlings were separated about two weeks after they emerged from egg sacs and were kept under the same conditions as their mothers.

Virgin F1 males (age 4–11 days from final molt) were used to set up the following treatment groups: 1) inexperienced males, i.e. males that did not have any contact with females, 2) males that were allowed to court females, 3) males that had mated with females shortly before fixation, and 4) males three days after mating.

2.2. Mating sequence

Courtship in *O. retusus* includes sequential behaviors starting with the male vibrating his opisthosoma. While approaching the female, his first pair of legs trembles and he plucks on the female's web. After contact with the tips of their first pair of legs, the partners assume the mating position, which we define as the end of courtship. The mating position is antiparallel with the female's prosoma above the male's prosoma (Schaible et al., 1986). In this position, the female contacts the surface of the male prosoma with her pedipalps and eventually positions her mouth region on the male's cephalic hump and releases and reingests saliva (Maes et al., 2004; Kunz et al., 2012).

2.3. Transmission Electron Microscopy

The cephalic hump of five to eight specimens per treatment group were dissected in 2.5% glutaraldehyde in 0.1 mol/l phosphate

the three section planes indicated. Orientation: left side: anterior, right side: posterior. (C) Schematic drawing of a type 1 gland of male *O. retusus*. CaC, canal cell; Ch, chelicera; CNS, brain; Cu, cuticle; Er, endoplasmic reticulum; Ey, eye; mCR, modified cephalic region; Mi, mitochondria; Mv, microvilli; N, nucleus; SC, secretory cell; Su, sulcus; V, vesicle. Modified after Michalik and Uhl (2011), reproduced with permission of John Wiley & Sons.

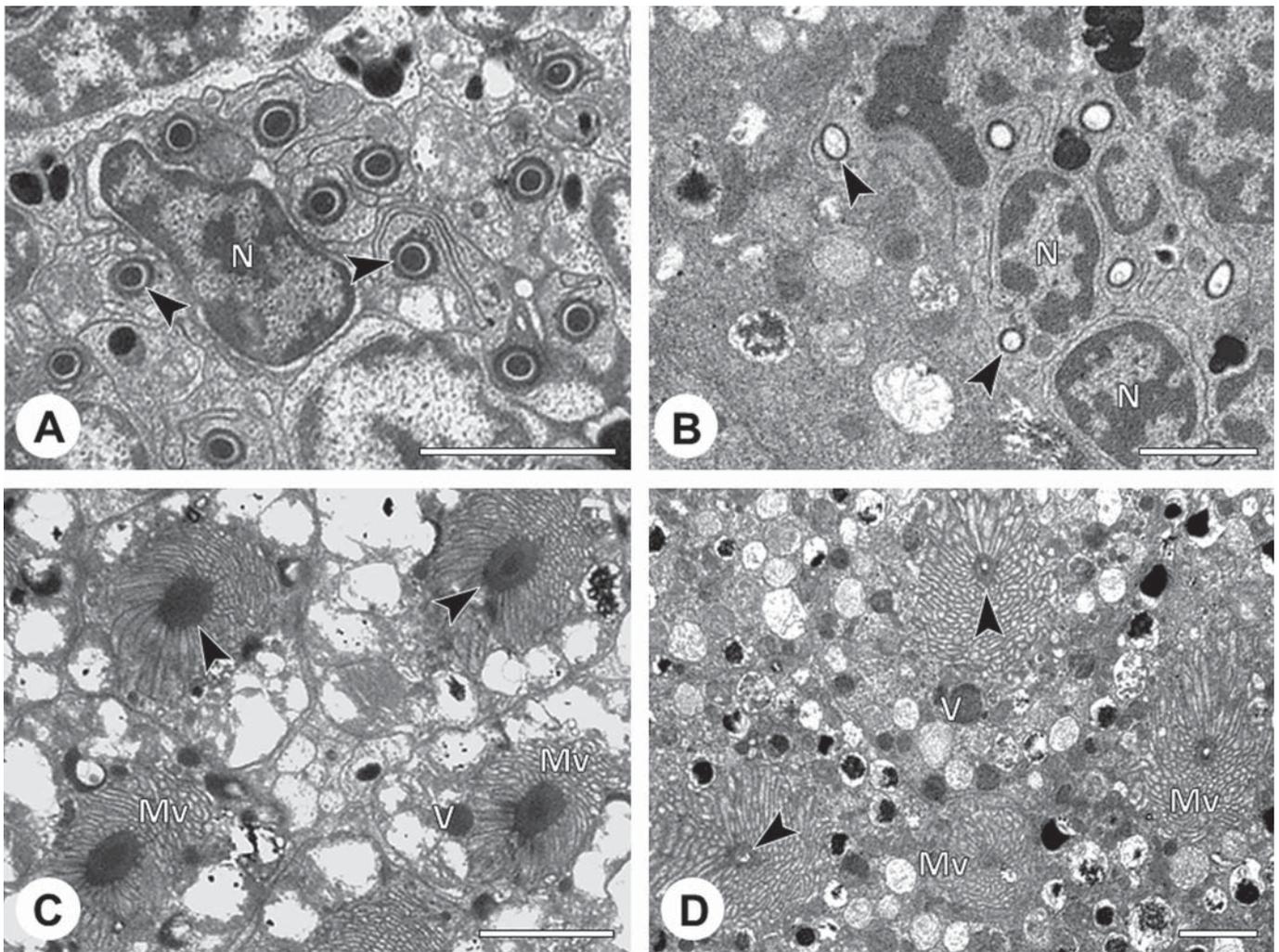


Fig. 2. Glandular units of male *Oedothorax retusus*. TEM. The conducting (A,B) and receiving canals (C,D) of the glandular units were classified according to the presence or absence of secretion. (A) Full conducting canals of an inexperienced male (arrowheads). (B) Empty conducting canals of a recently mated male (arrowheads). (C) Full receiving canals of an inexperienced male (arrowheads). (D) Empty receiving canals of a recently mated male (arrowheads). Scale bars 2 μm . Mv, microvilli; N, nucleus; V, vesicle.

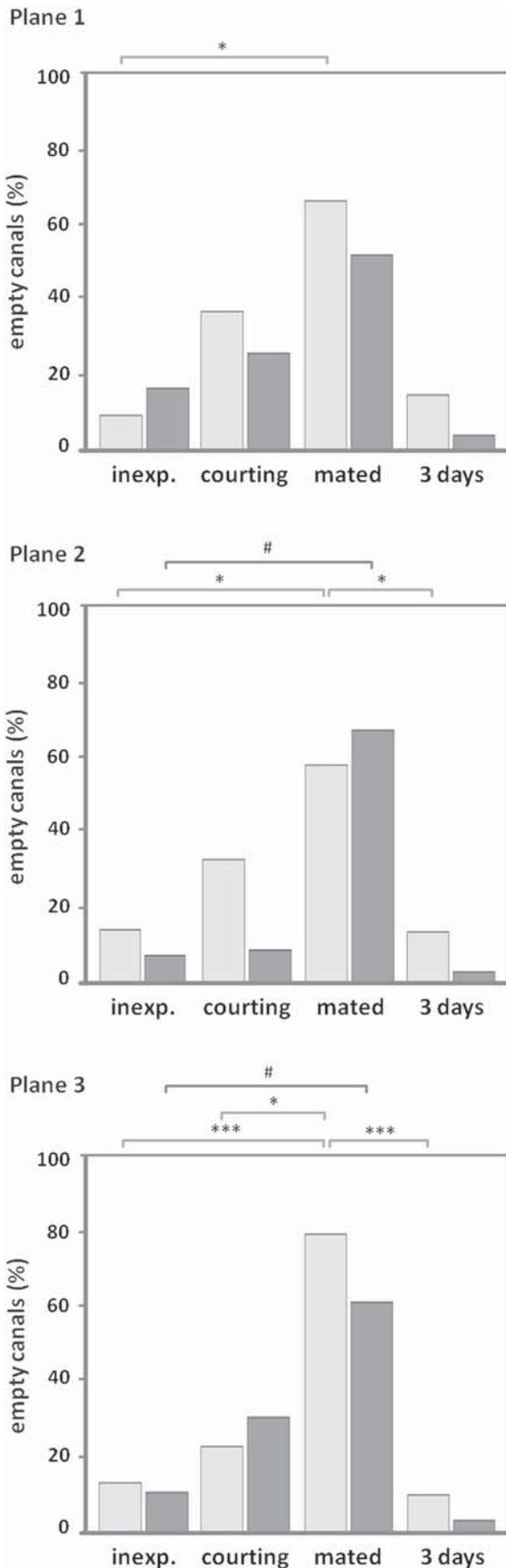
buffer (PB), pH 7.2, containing 1.8% sucrose. Prosomata were fixed in 2.5% glutaraldehyde in PB, washed, and post-fixed in 2% osmium tetroxide as described in Michalik and Uhl (2011). The material was then washed in PB, dehydrated in graded ethanol, and embedded in Spurr's resin (1969) or Agar resin (Agar Scientific, Essex, UK).

Ultrathin sections, obtained with a Leica UCT ultramicrotome (Leica Microsystems, Wetzlar, Germany) and Diatome diamond knife (Diatome AG, Biel, Switzerland), were routinely stained and observed with a JEOL 1011 transmission electron microscope (JEOL GmbH, Eching, Germany).

Table 1

Number of empty and full conducting and receiving canals per section plane through the male cephalic hump of *Oedothorax retusus*. Males differed in mating history prior to dissection and ultrastructural analysis.

Male treatments		Conducting canals (mean \pm SD)		Receiving canals (mean \pm SD)		Mean \pm SD Total
		Empty	Full	Empty	Full	
Section plane 1						
Inexperienced	N = 7	4.29 \pm 3.25	57.00 \pm 26.86	3.00 \pm 2.71	89.29 \pm 50.83	153.58 \pm 43.42
Courting	N = 5	32.60 \pm 44.39	28.60 \pm 23.52	4.60 \pm 7.06	67.00 \pm 62.27	132.80 \pm 35.39
Mated	N = 5	52.20 \pm 44.59	27.80 \pm 25.75	43.40 \pm 58.72	11.00 \pm 7.25	134.40 \pm 47.22
3 days after mating	N = 3	8.67 \pm 10.02	48.33 \pm 16.44	1.33 \pm 1.53	48.00 \pm 32.51	106.33 \pm 36.01
Section plane 2						
Inexperienced	N = 8	1.50 \pm 1.20	12.88 \pm 9.86	6.75 \pm 16.71	66.13 \pm 34.47	87.26 \pm 43.62
Courting	N = 6	13.67 \pm 18.81	25.50 \pm 18.23	39.50 \pm 60.52	68.33 \pm 41.14	147.00 \pm 31.71
Mated	N = 6	27.50 \pm 15.22	19.83 \pm 15.56	55.33 \pm 35.88	35.50 \pm 36.20	138.16 \pm 65.78
3 days after mating	N = 4	5.00 \pm 7.44	32.50 \pm 9.47	3.00 \pm 5.35	91.00 \pm 26.17	131.50 \pm 28.62
Section plane 3						
Inexperienced	N = 7	1.43 \pm 1.13	15.29 \pm 11.94	7.00 \pm 9.09	55.29 \pm 29.27	79.01 \pm 29.11
Courting	N = 7	8.00 \pm 11.33	17.86 \pm 16.78	15.86 \pm 19.96	41.86 \pm 32.59	83.58 \pm 28.46
Mated	N = 4/5	12.50 \pm 6.35	4.25 \pm 3.69	29.00 \pm 10.84	28.40 \pm 31.58	74.15 \pm 28.74
3 days after mating	N = 5	2.20 \pm 1.79	26.60 \pm 18.24	2.00 \pm 1.58	59.00 \pm 19.30	89.80 \pm 32.93



2.4. Investigation of glandular tissue

The glandular tissue of the focal species was described in detail in Michalik and Uhl (2011). We focused on type 1 glands (Fig. 1) since they dominated the glandular tissue. Each specimen was analyzed in three different section planes of the hump (Fig. 1). Section plane 1 was about 60 μm (number of glandular units (mean \pm SD): 137 ± 42), section plane 2 between 120 and 130 μm (number of glandular units: 122 ± 50) and section plane 3 between 190 and 200 μm (number of glandular units: 81 ± 28) from the top of the hump. All conducting and receiving canals of the glandular units were counted in each section plane and classified according to the absence or presence of secretion (empty, full, Fig. 2). Both types of canal regions can be found in each section plane because of the curvature of the cephalic hump (Fig. 1). Mean numbers of empty and full conducting and receiving canals per section plane and male treatment are shown in Table 1. Differences in percentages of empty conducting and receiving canals between treatments were calculated for the three section planes separately (Fig. 3).

2.5. Statistical analysis

Statistical analyses were performed using R. Data are given as arithmetic means \pm SD. For each section plane, we tested for differences between treatments (inexperienced, courting, mated, 3 days after mating) using generalized linear models (GLMs). Due to overdispersion (residual deviance was larger than degrees of freedom) we used quasibinomial and logit link function for the analyses. Pairwise post-hoc comparisons were performed using Tukey-HSD. Section planes were not pooled in order to avoid pseudoreplication of the data since some of the glandular cells may occur in more than one section plane.

3. Results

In section plane 1 both types of canals were observed in similar amounts (conducting canals: 51.27%; receiving canals: 48.73%). The percentages of empty conducting canals differed significantly between male treatments (GLM: $F = 5.42$, $p = 0.009$, Fig. 3, plane 1). Pairwise comparison showed that males that were dissected immediately after mating exhibited a significantly higher proportion of empty conducting canals compared to inexperienced males (Fig. 3, plane 1). The proportion of empty conducting canals was twice as much in males dissected immediately after mating compared to courting males, however non-significant (Fig. 3, plane 1). The proportions of empty receiving canals were not significantly different between treatments (GLM: $F = 3.35$, $p = 0.073$, Fig. 3, plane 1).

Section plane 2 mostly consists of receiving canals located in the reservoir region of the secretory cells (conducting canals: 25.87%, receiving canals: 74.13%). The proportions of empty conducting and receiving canals in section plane 2 differed significantly between treatments (GLM: conducting canals: $F = 5.09$, $p = 0.009$; receiving canals: $F = 4.15$, $p = 0.019$, Fig. 3, plane 2). Males that were dissected immediately after mating exhibited a significantly higher proportion of empty conducting canals compared to inexperienced males (Fig. 3, plane 2). The differences in the proportion of empty receiving canals of inexperienced males compared to mated males were nearly significant (Fig. 3, plane 2). The proportion of empty

Fig. 3. Percentages of empty conducting and receiving canals per section plane and treatment group (inexperienced, courting, mated, 3 days after mating) in *Oedothorax retusus*. Light gray = conducting canals, dark gray = receiving canals. Tukey-HSD tests were performed to test for differences between treatment groups of individual section planes. Asterisks mark significant differences ($^*0.01 \leq \alpha < 0.05$, $^{***}\alpha < 0.001$). Hash marks represent nearly significant differences ($\#0.05 < \alpha \leq 0.08$).

conducting canals was again twice as much in males dissected immediately after mating compared to courting males, however non-significant (Fig. 3, plane 2).

Section plane 3 also consists mostly of receiving canals (conduction canals: 25.75%, receiving canals: 74.25%). The proportions of empty conducting and receiving canals differed significantly between treatments (GLM: conducting canals: $F=9.327$, $p < 0.001$; receiving canals: $F=3.80$, $p=0.026$, Fig. 3, plane 3). Males that were dissected immediately after mating exhibited a significantly higher proportion of empty conducting canals compared to inexperienced males and males that were dissected 3 days after mating (Fig. 3, plane 3). The differences in the proportion of empty receiving canals of inexperienced males compared to mated males were again nearly significant (Fig. 3, plane 3). The proportion of empty conducting canals was threefold in males dissected immediately after mating compared to courting males (Fig. 3, plane 3). In all section planes, courting males did not differ significantly from inexperienced males and from those three days after mating (Fig. 3).

4. Discussion

We investigated the timing of the release and refilling of the glandular secretions of the male cephalic hump in *O. retusus*. We were able to show that shortly after mating - during which the cephalic structures of males are in direct contact with the female mouth region - 50–80% of conducting and receiving canals in all sections planes were found empty, whereas only 10–40% of canals were found empty in males that performed only courtship and were not in physical contact with the females. This difference was highly statistically significant for conducting canals in section plane 3 and demonstrates that secretions are released in a gustatorial context. When the female mouthparts are in contact with the cephalic hump, females can be observed to transfer saliva onto the hump and re-ingest it (Kunz et al., 2012). Very likely, female saliva liquefies the male secretion resulting in an uptake of a cocktail of fluid substances originating from both sexes.

It was previously suggested that the glandular tissue produces a volatile pheromone for female attraction (Vanacker et al., 2003; Huber, 2005). If a volatile pheromone were emitted, we would expect the cephalic glands to be most active during courtship in order to attract females to mate. However, this was not the case. Compared to inexperienced males and recovered males, courting males showed more empty canals on average, however non-significantly so. Possibly, courting males release some secretion prior to contact with the female mouthparts to present some substances that can be readily accessed by the female as soon as she achieves mating position.

Inexperienced males and males three days after mating exhibited only 3–16% of empty canals, highly significantly different from the situation in males that were dissected immediately after mating (conducting canals, section planes 2 and 3). Obviously, refilling of the canals is accomplished at the latest after three days. Why some of the canals were found empty even in inexperienced males is unclear. An explanation could be that the slight mechanical pressure applied when dissecting out the small area of the hump resulted in a certain proportion of secretion release in all specimens. If so, the relative difference in filling degree between groups remains meaningful. Pairwise post-hoc tests revealed stronger treatment effects for conducting canals compared to receiving canals. Since conducting canals are close to the exit and small in diameter, they can be emptied more quickly compared to the glandular reservoirs.

Nuptial gifts such as secretory substances are generally considered to function either as male mating efforts or paternal investments that have evolved under sexual selection (Andersson,

1994; Vahed, 1998, 2007; Gwynne, 2008). Detailed investigations on the effect of cephalic secretions of *O. retusus* on mating and paternity demonstrated that they are not a prerequisite for a successful mating attempt (Kunz et al., 2012). However, mating probability was significantly higher for males from which uptake of secretions by the female was possible compared to males whose cephalic region was covered by a thin layer of paint (Kunz et al., 2012). There was no detectable effect on relative paternity after the female had mated with a covered and an uncovered male (Kunz et al., 2012). Therefore, the secretions can be considered to function as a male mating effort. Gift-giving males are accepted with higher probability and will therefore have higher overall mating success and a higher overall number of offspring compared to non-gift giving males. Paternity success is further secured in *O. retusus* by the application of a mating plug to the female genital opening (Uhl and Busch, 2009).

Male cephalic structures are very diverse in erigonine spiders, strongly suggesting that they all evolved under sexual selection (Eberhard, 1985, 1996; Vanacker et al., 2003; Huber, 2005). The linyphiid mating position in which the partners are in an antiparallel position with the female mouthparts above the male ocular region (von Helversen, 1976), can be considered a preadaptation to gustatorial courtship via cephalic structures. We find cephalic modifications in about 80 erigonine genera (of an estimated number of 300 genera) (Hormiga, 2000; Miller, 2007). A preliminary phylogenetic analysis suggests multiple independent origins of cephalic modifications (Lopardo and Uhl, unpublished). It remains to be investigated whether all cephalic modifications evolved in the same context, namely gustatorial courtship.

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**Short-term nutritional limitation affects mating behaviour and
reproductive output in dwarf spiders**

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Short-term nutritional limitation affects mating behaviour and reproductive output in dwarf spiders

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Short title: Mating and reproduction under nutritional limitations

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Mating decisions and mating behaviour can vary considerably depending on individual experience and mate and food availability. In particular, foraging history has been demonstrated to impact strongly on female mate choice. Here, we applied short-term dietary restrictions to adult female spiders that were well fed during the juvenile stage in an effort to understand whether and how brief periods of food shortage can influence male and female mating decisions and mating behaviour. In order to assess how strongly responses can vary within a genus, we conducted the same experiment on two closely related dwarf spider species *Oedothorax retusus* and *O. apicatus*. Males of both species offer secretion to the female from glandular tissue in their prosoma during courtship and mating. Females were subject to food shortage over a period of three weeks ('low-diet' treatment, LD) or fed regularly ('high-diet' treatment, HD). We compared courtship probability, mating probability/behaviour, and reproductive output between mating trials between dietary groups and species. In both *Oedothorax* species, females in the LD treatment were less likely to mate and more aggressive towards males. In *O. retusus*, females were more likely to show gustatory behaviour and had reduced copulation duration in the LD group, but not in *O. apicatus*. In both species, LD females produced egg sacs significantly later than HD females. Hatching success between treatments was similarly high in *O. retusus* that produced a lower number of eggs under the LD treatment. In *O. apicatus*, however, egg number was not reduced in the LD treatment but hatching success was extremely low. Our study shows that short phases of dietary restriction during the adult stage can strongly affect mating behaviour and reproductive output with differences between closely related species.

Introduction

Mating behaviour is influenced by many factors like individual experience and condition, age, or environmental conditions such as food availability and foraging history (Gray 1999; Ortigosa & Rowe 2002; Hebets et al. 2008; Eraly et al. 2009; Immonen et al. 2009; Adams & Morse 2014). Mating behaviour further depends on local mate availability and the quality of the available mating partners resulting in choosiness of either sex (Andersson & Iwasa 1996). Several studies emphasize that mating preferences of both sexes vary during a lifetime (Brown & Kuns 2000; Burley & Foster 2006; Fisher & Rosenthal 2006; Howard & Young 1998), and change depending on an individual's own condition (Cotton et al. 2006; Holveck & Riebel 2010). Consequently, optimal mate choice in both sexes is a compromise between mate qualities, cost of mating, and risk of losing an opportunity to mate with another partner (Elgar & Nash 1988; Bonduriansky 2001; Bleu et al. 2012).

Nutritional condition of both mating partners plays an important role in mate choice decisions (Schneider & Elgar 2002; Engqvist & Sauer 2003; Arnqvist & Rowe 2005; Fisher & Rosenthal 2006; Garbutt & Little 2014). Mating decisions of males and females may be influenced on a fine temporal scale depending on momentary food abundance, which may intensify conflicts between the sexes over mating in a particular time window (Fisher & Rosenthal 2006). Generally, well-fed individuals were shown to profit in several respects compared with food-limited individuals: higher mating probability, higher number of matings, longer copulation durations, and higher reproductive output (Engqvist & Sauer 2003; Hebets et al. 2008; Eraly et al. 2009; Albo et al. 2012). In species such as crickets, scorpionflies, beetles, and spiders, male body condition was shown to affect male courtship, female mate choice, and mating success (Gwynne 1993; Mappes et al. 1996; Andrade & Mason 2000; Ahtiainen et al. 2002; Kotiaho 2002; Hunt et al. 2005; Hoefler et al. 2008; Engqvist 2009; Lomborg & Toft 2009; Albo et al. 2012). Low nutritional condition of a given male can result in high susceptibility to diseases or in low sperm quality and/or quantity (Giaquinto et al. 2010). The higher the resource limitation, the more likely it is that males discriminate between females of different condition (Gwynne 1993; Byrne & Rice 2006). Males may assess a female's fecundity directly through visual or tactile mechanisms, and perform mate choice via proxies of female fecundity e.g. condition, size or weight (Gwynne 1993; Bonduriansky 2001; Byrne & Rice 2006; Barry 2010). Female mating decisions can be affected not only by the male phenotype but may considerably depend on her own feeding history (Engqvist & Sauer 2003; Fisher & Rosenthal 2006; Engqvist 2009). For example, females in poor condition can increase their fecundity by preferentially mating with males that offer nuptial gifts (Immonen et al. 2009; Fox & Moya-Laraño 2009) or by simply consuming their mating partner (Andrade 1998; Herberstein et al. 2002; Barry 2010; Barry et al. 2008, 2010; Berning et al. 2012). In the praying mantid *Pseudomantis albobimbrata* females in low body condition are more likely to perform precopulatory sexual cannibalism (Barry 2010). However, in spiders it was shown that even

hungry females exhibit mate choice in that they spare high quality males but cannibalize low quality males before or after copulation (Elgar & Nash 1988).

Here, we investigate how a brief period of food shortage in adult females impacts on their courtship activity, mating probability, mating behaviours and reproductive output and whether males differentiate between females with short term differences in feeding history. We compare these parameters between two dwarf spiders *Oedothorax retusus* and *O. apicatus* that are considered sister species in a recent phylogenetic analysis (Lopardo & Uhl 2014), which lends them particularly to investigating to what degree responses to short term food limitation are fixed in closely related species. In both species, the male prosoma is elevated in a species-specific way and possesses glandular tissue, which produces secretions that are taken up by the female during courtship and mating (Michalik & Uhl 2011; Kunz et al. 2013). In *O. retusus*, the secretion was found to increase mating probability and to stimulate egg production (Kunz et al. 2012). Consequently, we expect that food limited females would be more prone to forage for these secretory nuptial gifts.

Methods

Study species

Oedothorax retusus and *O. apicatus* are European dwarf spiders (Linyphiidae, Erigoninae). *O. retusus* occurs in wet habitats (salt marshes, riverbanks), whereas *O. apicatus* is common on cultivated, agricultural areas. For the present study, adult females of *O. retusus* were collected from the banks of the River Rhine, near Bonn, Germany (50°43'00.26"N, 7°08'25.11"E – 50°42'57.40"N, 7°08'36.70"E) in April/September 2010-2012. Adult *O. apicatus* females were collected from an agricultural field situated between Bonn and Siegburg, Germany (50°45'19.16"N, 7°09'11.11"E - 50°45'17.76"N, 7°09'07.20"E) in September 2011 and April/September 2012. Offspring were individually reared in the lab under controlled conditions (23/17 °C (day/night), 70% humidity) in 25 ml plastic containers equipped with a moist layer of gypsum. Spiderlings were raised separately from egg sacs and fed *ad libitum* on springtails (*Sinella curviseta*) until the subadult stage (Kunz et al. 2012). Subadults were fed on four to six fruit flies (*Drosophila melanogaster*) per week and were checked weekly for the final moult to adulthood. Mating experiments were performed between September 2010 and November 2012.

Experimental setup

We randomly assigned freshly moulted, virgin females to 'high-diet' (HD) or 'low-diet' (LD) treatments. During three weeks after the final moult, HD females were fed four to six fruit flies per week, while LD females were fed a maximum of three flies in three weeks. In the lab, females of *O. retusus* and *O. apicatus* live up to 12 month after their final moult, while males have a lifespan of up

to 8 month. Thus, 3 weeks food limitation is a short time period in those species. Mating trials with a total of 371 virgin females and 371 virgin males (*O. retusus*: HD: n= 116, LD: n= 78; *O. apicatus*: HD: n= 91, LD: n= 86) were staged. At the day of the mating experiment, females were on average 21.6±3.8 days (*O. retusus*: HD 22.4±4.7, LD 22.1±2.6; *O. apicatus*: HD 21.3±4.1, LD 20.3±2.3), and males 22.0±3.9 days old (*O. retusus*: HD 22.8±5.0, LD 21.8±2.7; *O. apicatus*: HD 22.3±4.1, LD 20.9±2.7). We used female weight at the day of the mating trial as a proxy for female fecundity. LD females were significantly lighter than HD females in *O. retusus* (weight (mg): HD 3.62±0.46, n= 21; LD 1.86±0.25, n= 25; T-test: T= 15.72, p< 0.001) and *O. apicatus* (HD 3.84±0.57, n= 13; LD 1.90±0.20, n= 48; T-test: T= 12.04, p< 0.001). Mating trials started by gently transferring a male into the container of the female. We compared male courtship probability, probability of female aggressive behaviour (attacking and chasing the male), insertion probability (insertion of one pedipalp into one female genital opening), duration of pedipalp insertion, whether the female salivated onto the male's prosoma for uptake of secretions, and who ended the insertion (see Kunz et al. 2012 for more detailed definition of parameters). After the mating trials, all females were fed on a regular, weekly feeding schedule with four to six fruit flies until oviposition. As parameters of reproductive output we recorded oviposition latency, weight of the egg sac (Sartorius ME5 micro scale: 5g capacity, 1 µg readability, ± 1µg reproducibility), egg number, egg mass (weight of the egg sac/number of eggs), and hatching success (percentage of hatched spiderlings). We compared parameters between mating trials with HD and LD females within and between species.

Statistical analysis

Statistical analyses were performed with IBM SPSS Statistics 22.0. Data are given as arithmetic mean±SD for normally distributed data, or median [interquartile range] for non-normally distributed data. All tests were performed two-tailed.

Results

Courtship and mating probability

Male courtship probability in *O. retusus* males was not significantly different when exposed to HD and LD females (Table 1). In *O. apicatus*, however, males were significantly more reluctant to court LD females (Table 1). Differences in courtship probability between species were not significant (Chi²-tests: HD: n= 207, Chi²= 0.60, p= 0.440; LD: n= 164, Chi²= 0.02, p= 0.885). In both species, LD females were significantly more likely to show aggressive behaviour towards the male than HD females (Table 1) but cannibalization did not occur. Mating probability of first insertions depended significantly on female feeding regime in both species and was less likely with LD females (Table 1, Fig. 1). Mating probabilities were not significantly different between species (Chi²-tests: HD: n= 207, Chi²= 0.73, p=

0.392; LD: n= 164, $\chi^2= 1.60$, $p= 0.206$). In both species, it was generally the males that were reluctant to mate; however, the difference between treatments was not significant (Table 1), and most likely a consequence of female aggressive behaviour. Female aggressive behaviour significantly influenced insertion probabilities in both HD and LD treatments. First insertions were less likely if females behaved aggressively in HD and LD treatments of both species (insertion probability: *O. retusus* HD: aggressive females 50 %, 2 of 4; nonaggressive females 87.5%, 98 of 112; $\chi^2= 4.57$, $p= 0.033$; *O. apicatus* HD: no cases of aggressive behaviour, n= 91; *O. retusus* LD: aggressive females 28.6%, 4 of 14; nonaggressive females 71.9%, 46 of 64; $\chi^2= 9.36$, $p= 0.002$; *O. apicatus* LD: aggressive females 55%, 11 of 20; nonaggressive females 78.8%, 52 of 66; $\chi^2= 4.43$, $p= 0.035$). In *O. retusus*, the duration of the first insertion was significantly reduced with LD females, and LD females applied saliva onto the male's prosoma with significantly higher probability compared to HD females (Table 1). None of these parameters was significantly different between LD and HD groups in *O. apicatus*. Most of the matings entailed a further insertion with the other pedipalp. However, the probability of a second insertion to occur did not differ significantly between feeding regimes (Table 1). Aggressive behaviour by the female rarely occurred prior to a second insertion (1-8%) and was not significantly different between feeding regimes of both species (Table 1). In both species, the duration of the second insertion was not significantly different between feeding regimes (Table 1). In *O. retusus* duration of the second insertion was significantly different from the first insertion duration in the LD treatment, but not in the HD treatment (paired t-test: HD: n= 92, $T= 1.25$, $p= 0.215$, LD: n= 47, $T= -2.06$, $p= 0.045$; see Table 1 for data). In *O. apicatus* duration of the second insertion was not significantly different from first insertion duration in both treatments (Wilcoxon-test: HD: n= 76, $Z= -1.10$, $p= 0.271$, LD: n= 60, $Z= -0.11$, $p= 0.909$; see Table 1 for data).

Reproductive output

Oviposition latency was significantly different between female feeding regimes in both species. In *O. retusus*, LD females took on average 23 days longer than HD females and in *O. apicatus* LD females took 26 days longer than HD females (Table 1). Furthermore, female feeding status had a significant effect on the weight of the egg sac with egg sacs of LD females being significantly lighter than those of HD- females in both species (*O. retusus*: 37.5% lighter, *O. apicatus* 15.3% lighter, Table 1, Fig. 2). However, single egg mass did not differ significantly between feeding regimes of *O. retusus* or *O. apicatus* females (Table 1). Lower egg sac weight resulted from significantly fewer egg numbers (-55.5%) in LD *O. retusus* females whereas the 15.3% reduction in egg number in *O. apicatus* females is not statistically significant (Table 1). In *O. retusus*, the percentage of hatched spiderlings was not significantly different between female feeding regimes but in *O. apicatus*, LD females suffered from a significant decrease in hatching success (Table 1).

Table 1. Mating behaviours and parameters of reproductive output in mating trials of *Oedothorax retusus* and *O. apicatus* with ‘high-diet’ (HD) or ‘low-diet’ (LD) females. Data are given as events (proportions), mean±standard deviation, and median [interquartile range]. Statistical analysis was performed using Chi²-test, Mann-Whitney-U-test (#) and T-test (*). Significant values are marked in bold, “ marks a missing case in the *O. retusus* LD treatment because of a malformed male pedipalp.

	<i>O. retusus</i>										<i>O. apicatus</i>									
	‘high-diet’					‘low-diet’					‘high-diet’					‘low-diet’				
	n	Mean/Median	N	Events	Z [#] , T [*] , Chi ²	P	n	Mean/Median	N	Events	Z [#] , T [*] , Chi ²	P	n	Mean/Median	N	Events	Z [#] , T [*] , Chi ²	P		
male courts	116	113:3 (97.4%)	78	73:5 (93.6%)	1.73	0.189	91	90:1 (98.9%)	86	80:6 (93.0%)	4.02	0.045								
first insertion																				
female aggressive	116	4:112 (3.5%)	78	14:64 (20.6%)	11.65	0.001	91	0:91 (0.0%)	86	20:66 (23.3%)	23.86	<0.001								
insertion probability	116	100:16 (86.2%)	78	50:28 (64.1%)	13.00	<0.001	91	82:9 (90.1%)	86	63:23 (73.3%)	8.48	0.004								
insertion duration (sec)	100	206.5±64.0	50	168.8±64.5	3.39*	0.001	82	369 [265]	63	365 [129]	-0.03#	0.975								
female salivates	75	24:51 (32.0%)	46	24:22 (52.2%)	4.85	0.028	70	39:31 (55.7%)	60	38:22 (63.3%)	0.78	0.378								
insertion ended by (male:female)	100	90:10 (90.0%)	50	44:6 (88.0%)	0.14	0.708	82	77:5 (93.9%)	63	60:3 (95.2%)	0.12	0.727								
second insertion																				
female aggressive	100	1:99 (1.0%)	49#	2:47 (4.1%)	1.58	0.208	82	3:79 (3.7%)	63	5:58 (7.9%)	1.25	0.263								
insertion probability	100	92:8 (92.0%)	49#	47:2 (95.9%)	0.20	0.658	82	76:6 (92.7%)	63	60:3 (95.2%)	0.40	0.527								
insertion duration (sec)	92	195.0±71.7	47	189.2±65.3	0.47*	0.641	76	391 [351]	60	371 [224]	-1.00#	0.318								
female salivates	72	24:48 (33.3%)	41	17:24 (41.5%)	0.75	0.387	64	43:21 (67.2%)	49	37:12 (75.5%)	0.93	0.335								
insertion ended by (male:female)	92	87:5 (94.6%)	47	44:3 (93.6%)	0.05	0.820	76	69:7 (90.8%)	60	59:1 (98.3%)	3.45	0.063								
oviposition latency (sec)	79	9 [30]	19	32 [38]	-3.60#	<0.001	68	31 [31]	35	57 [38]	-4.75#	<0.001								
weight egg sac (mg)	76	2.83±0.83	17	1.77±0.83	4.72*	<0.001	68	2.22±0.56	36	1.88±0.65	2.81*	0.006								
egg mass (mg)	66	0.13 [0.04]	5	0.15 [0.09]	1.79#	0.074	51	0.14 [0.05]	24	0.12 [0.05]	-0.83#	0.407								
egg number	66	22.9±8.6	5	10.2±6.2	3.24*	0.002	51	16.1±5.8	24	14.6±5.6	1.04*	0.302								
hatching success (%)	66	54 [95]	5	80 [92]	-0.29#	0.773	51	100 [77]	24	0 [88]	-3.33#	0.001								

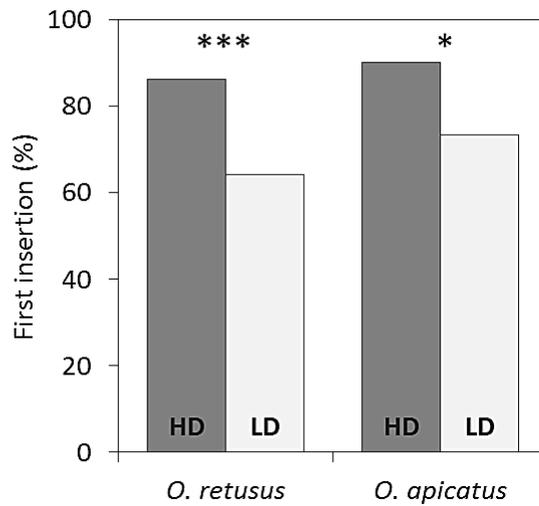


Figure 1. — Probability of mating (first insertions) in *Oedothorax retusus* and *O. apicatus* mating trials with ‘high-diet’ (HD) and ‘low-diet’ (LD) females. Asterisks mark significant differences between feeding regimes (* $0.01 < \alpha \leq 0.05$, *** $\alpha \leq 0.001$).

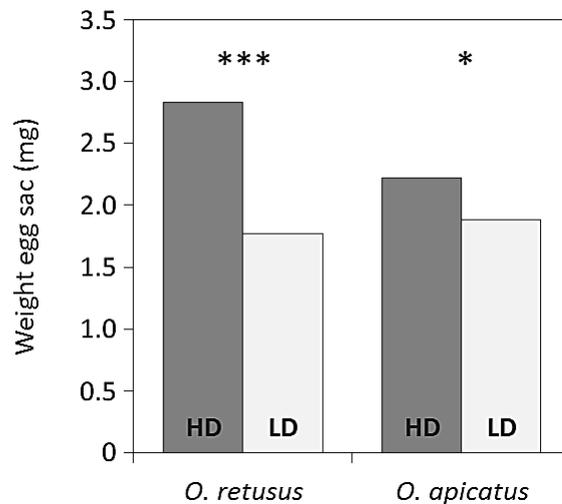


Figure 2. — Weight of egg sacs (in mg) in *Oedothorax retusus* and *O. apicatus* mating trials with ‘high-diet’ (HD) and ‘low-diet’ (LD) females. Asterisks mark significant differences between feeding regimes (* $0.01 < \alpha \leq 0.05$, *** $\alpha \leq 0.001$).

Discussion

We analysed the effect of short term feeding limitation in the female on mating behaviour and reproductive output in two closely related dwarf spider species. In both species, mating was less likely with females of the ‘low-diet’ treatment (LD) compared to the ‘high-diet’ treatment (HD) which seems to be due to increased aggression by LD females towards their potential mates and partly to

male reluctance to court LD females. Short term feeding limitation resulted in postponed oviposition and reduced weight of the egg sacs in both species. The two species, however, differed with respect to effects of feeding treatment on copulation duration, gustatorial courtship behaviour and reproductive output.

When potential mating partners meet, mating probability depends on female receptivity and on mate preferences of both sexes that often vary greatly (Howard & Young 1998; Brown & Kuns 2000; Burley & Foster 2006; Adams & Morse 2014). Under food shortage, female receptivity can be reduced to avoid costly courtship and mating activity (Perry et al. 2009). In carnivorous species such as spiders, females may consume the male before or after mating depending on their hunger level (feeding opportunism hypothesis, Andrade 1998). In the praying mantid *Pseudomantis albofimbriata*, cannibalistic females improve their body condition and produce heavier egg sacs compared to non-cannibalistic females (Barry et al. 2008). In both *Oedothorax* species investigated here, female aggression before mating increased about 20 fold in food-limited females, suggesting that females may have tried to cannibalize the male. Deadly attacks, however, did not take place despite the small mating arena. Low cannibalism probability may be due to the fact that males and females in *Oedothorax* are of similar size in contrast to most other sexually cannibalistic species in which males are much smaller and thus more easily overcome by the female (Andrade 1998). Possibly, LD *Oedothorax* females react faster to any movement in their webs and thereby discriminate mates from potential prey later than HD females.

Interestingly, despite an increase in aggressive behaviour, a high percentage of females nevertheless engaged in matings even under the food deprived condition (64% *O. retusus*, 73% *O. apicatus*). Consuming nuptial gifts that add to female condition may compensate for costly matings or food deprivation (reviewed in Vahed 2007). In a study on the effect of male cephalic secretions in *O. retusus*, females that received greater amounts of secretions from the males produced more offspring (Kunz et al. 2012). Since LD females were significantly more likely to salivate onto the gift producing male cephalic structures in the present study we assume that these females tried to forage for male cephalic secretions. Increasing gustatorial activity was not present in *O. apicatus* for which the function of the cephalic secretions remains to be investigated. However, our study suggests that the functions of the gustatorial secretions may differ even between closely related species.

Although mate choice has mainly been attributed to females, variation in female quality, low mate search costs, and sperm limitation can select for the evolution of male mate choice, which seems to be more common than previously assumed (Bonduriansky 2001; Bateman & Fleming 2006; Barry 2010; Barry & Kokko 2010; Edwards & Chapman 2011; Lombardo et al. 2012; Adams & Morse 2014). Barry (2010), and Barry et al. (2010) showed that males in the praying mantid *Pseudomantis*

albofimbriata are attracted differently to females that vary in conditions, thereby choosing females that lend the highest potential reproductive success (for other insect species see: Bonduriansky 2001). In our comparative study, males were generally the sex that was reluctant to mate, however, only in *O. apicatus*, male courtship probability was significantly less likely when the males were confronted with LD females. However, rejecting a female can reduce male reproductive success considerably, which is one of the main reasons why theoretically male mate choice should not necessarily evolve (Barry et al. 2010; Barry & Kokko 2010). In our study, however, *O. apicatus* male investment in mating activity including gustatorial courtship is very likely lost with short-term food deprived females since the majority of these females failed to produce viable offspring. Consequently, nutritional limitation has a particularly strong impact on female reproductive output in *O. apicatus* despite the fact that food limitation was only brief and occurred before copulation. Why LD *O. apicatus* females cannot compensate short-term nutritional limitation during the two months post mating period remains to be investigated. However, our finding corresponds with theoretical models demonstrating that male choosiness is expected to evolve particularly in species with high variation in female quality as well as high mate availability (Barry & Kokko 2010).

It has been known from many arthropod species that a low female nutritional status can affect reproduction by an increase in oviposition latency or a decrease in egg number or egg mass (Gwynne 1988; Vahed 1998, 2007; Simmons et al. 1999; Simmons 2001; Bergström & Wiklund 2002; Engqvist 2007). Since food limitation in arthropods is assumed to occur frequently under natural conditions (Denno & Fagan 2003; Fagan & Denno 2004), such effects are expected to strongly shape reproductive behaviour. However, the degree to which short-term resource limitation affects reproductive output in both *Oedothorax* species is unexpectedly high. In our study LD females that were food limited for three weeks before mating, postponed oviposition for about the same time. More importantly, regular feeding after mating could not compensate for the phase of food stress prior to mating. LD females suffered from severe long-term costs in several ways: in both species, females produced egg sacs that were significantly lighter, compared to the HD treatments. *O. retusus* LD females produced only half the number of eggs and offspring compared to HD females while in *O. apicatus* LD females reduced the number of eggs laid only slightly but suffered from an extremely low hatching success.

Our study demonstrates that short phases of food deprivation - as regularly occur in nature - can have unexpectedly strong immediate effects on mating and subsequent effects on reproductive output. Overall, our study on the dwarf spider species *O. retusus* and *O. apicatus* shows that females in low condition are more aggressive towards courting males, and that males are more likely to decide against females with expected low reproductive output at least in *O. apicatus*. Reproductive success with short-term food deprived females was low in both species, which demonstrates the

severe long-term impact of food limitation on fecundity. Our study further demonstrates that effects of short term feeding limitations cannot easily be generalized even between closely related species.

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A spider mating plug: origin and constraints of production

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A spider mating plug: origin and constraints of production

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Males can increase their reproductive success by mechanically hindering females to mate with subsequent males. Research on mating plugs so far has focused on the fitness consequences and demonstrated that plug size can strongly determine its efficacy. Here, we explore: (1) the site of plug production in the erigonine spider *Oedothorax retusus*; and (2) whether males are limited in the production of plug material when mating with three females in succession. Micro-computed tomography, histological and ultrastructural sections demonstrate that the plug material is produced in a massive gland inside the sperm transfer organs of the male, the pedipalps. The glandular lumen is connected with the tube-like spermophore almost at its blind end. Probably, a reservoir of plug material is built up at the end of the spermophore and released after sperm transfer onto the female genital opening. Since not all males applied a large plug during their first mating, there was no significant decline in plug size over the course of the three successive matings. However, the size of the first plug significantly affected the size of the following plug. We discuss these findings in the light of plug limitation and mate choice. © 2014 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2014, **113**, 345–354.

ADDITIONAL KEYWORDS: 3D-reconstruction – gland – male mate choice – micro-computed X-ray tomography – monopolization – sexual selection – trade-off – ultrastructure.

INTRODUCTION

Females of many animal species mate with more than one male, and store sperm within their genital tract for later use. Consequently, sperm from different males compete over fertilization of the eggs (Parker, 1970). Thus, any trait that reduces or impedes further matings of the female is advantageous for the first male to mate. Behavioural adaptations such as guarding the female until oviposition are very time consuming and often restrict the male to a single mating. Therefore, traits that prevent the female from remating without the necessity of the male's presence provide a high selective advantage. Accordingly, mating plugs that block or at least impede access to female genitalia have evolved many times indepen-

dently in many animal groups (Drummond, 1984; Eberhard, 1996; Birkhead & Møller, 1998; Simmons, 2001; Oh & Hankin, 2004; Peretti, 2010; Uhl, Nessler & Schneider, 2010).

Mating plugs can consist of ejaculates, amorphous substances and male somatic or genital parts that are left behind on and in the female genital tract (see references above) and can also be produced by females (Aisenberg & Eberhard, 2009; Aisenberg & Barrantes, 2011). Even the whole male body can function as a mating plug (Knoflach & Benjamin, 2003). Although these materials are generally suspected to serve paternity protection by mechanically hindering rival males to mate (Simmons, 2001), they may serve different or multiple purposes, most of which are also related to conflicts within or between the sexes. They may lower female attractiveness to rival males (Baer *et al.*, 2000) or female receptivity (Shine, Olsson & Mason, 2000), may prevent sperm dumping by the female, may allow female choice by estimating male ability in protecting sperm or in

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removing plugs (Eberhard, 1996) or may simply prevent sperm backflow.

Spiders are highly suitable for investigating general post-copulatory processes (Herberstein *et al.*, 2011) and particularly the adaptive value and efficacy of mating plugs. A recent review reported a tentative 200 species showing either amorphous material (about 70%) or stuck genital male structures (about 30%) on the female genital area (Uhl *et al.*, 2010). The central role mating plugs seem to have in spider reproductive biology may be due to the fact that in the majority of female spiders the copulatory openings are separate from the openings used for egg laying (Foelix, 2011). Consequently, oviposition is not impeded by the material deposited on the copulatory openings.

Whereas the origin of broken off male genitalia is obvious (e.g. Uhl, Nessler & Schneider, 2007), the production sites of the amorphous secretions are less clear. The secretion can be transferred by any structure that is in contact with the female genital region during or immediately after mating. Potential production sites of amorphous plugs are the pedipalps that function as interim sperm storage sites and sperm transfer organs in male spiders (Suhm, Thaler & Alberti, 1996). The material may also be produced in the male genital tract and consist of a mixture of sperm and secretions (Huber, 1995). In some species, the material was suggested to be produced in the mouth region of the male, who seems to load the tips of the pedipalps after mating and transfers the substance during a second genital contact (Braun, 1956). Alternatively, the plug material may be produced fully or partly by the female, very likely as a means of cryptic female mate choice (Knoflach, 1998; Aisenberg & Eberhard, 2009).

To date, there is only a single study that investigated the production site of the amorphous secretion in detail demonstrating that in the amaurobiid spiders *Amaurobius fenestralis* (Stroem, 1768) and *A. ferox* (Walckenaer, 1890), the plug material is produced in a gland located inside the male pedipalp (Suhm *et al.*, 1996). The glandular material is released at the base of the sperm transferring structure, the embolus. The gland is not connected to the tube-like spermophore in which sperm is stored inside the pedipalp. Whether a rival male can overcome a plug has not been investigated for *Amaurobius*. That plugs indeed can be removed has been shown in a salticid, an agelenid and a linyphiid spider (Jackson, 1980; Matsumoto, 1993; Uhl & Busch, 2009). In these species, plug size determines effectiveness with small plugs being more likely removed than complete, large plugs.

For the dwarf spider *Oedothorax retusus* (Linyphiidae: Erigoninae) we have clear evidence that the

amorphous secretion functions as a copulation barrier and thus as a means to secure paternity (Uhl & Busch, 2009). Males have a high chance of monopolizing the female if they are allowed to perform two copulations by which both of her copulatory openings can be plugged (Richter N & Uhl G, unpublished). Apart from size as a predictor of plug efficacy the age of the mating plug also plays an important role: successful remating of the plugged female with a rival male is particularly high shortly after the first mating suggesting that the material requires hardening (Kunz, Witthuhn & Uhl, 2014). Large, hardened plugs are highly effective and remain so over weeks, and even survive oviposition (Kunz *et al.*, 2014).

In order to explore further the mechanisms that shape the efficacy of mating plugs and thus the strength of sexual selection we aim to clarify the site of plug production and whether males are limited in producing the secretion that forms the mating plug. First, we scrutinize the male pedipalp as a potential production site of the mating plug material using semithin and ultrathin sectioning as well as X-ray micro-computed tomography (micro-CT). Secondly, we investigate the constraints involved in producing plug material by performing mating experiments in which males mate consecutively with three virgin females. We hypothesize that plug sizes are large in first matings and smaller in consecutive matings if males are limited in plug production. This study complements previous investigations on the function of the mating plug in *O. retusus* (Uhl & Busch, 2009; Kunz *et al.*, 2014) leading to the most comprehensive understanding of the production and adaptive value of an amorphous mating plug in spiders.

METHODS

Oedothorax retusus (Westring, 1851) (Erigoninae: Linyphiidae) were raised in the laboratory from egg sacs of females caught near Bonn, Germany, along the banks of the river Rhine (see Kunz, Garbe & Uhl, 2012 for details).

PLUG PRODUCTION SITE

In *O. retusus*, observation of the mating behaviour suggested that the secretion must be produced inside the male secondary copulatory organs, the paired pedipalps. Pedipalps are loaded with sperm after the final molt to adulthood. During copulation, a pedipalp is connected to one of the paired female copulatory openings and spermatozoa are transferred into one of her spermathecae where they are stored until egg laying. In 2011, pedipalps of 12 adult males ($N = 24$) were fixed overnight in 2.5%

glutardialdehyde, transferred to phosphate buffer (0.1 M, pH 7.2), post-fixed in buffered (2%) osmium tetroxide (OsO_4) and dehydrated in graded series of ethanol. For light- and transmission electron microscopy 20 palps were embedded in Spurr's resin (Spurr, 1969). Sections (semithin and ultrathin) were made with a Diatome diamond knife on a Leica UC6 and UCT ultramicrotome. Semithin sections (700 nm) were stained after Richardson, Jarrett & Finke (1960). Ultrathin sections (50–70 nm) were stained with lead citrate and uranyl acetate according to Reynolds (1963) and investigated with a JEOL JEM 1011 transmission electron microscope operating at 80 kV. Images were obtained with an Olympus Mega View III digital camera using the iTEM software (Olympus SIS).

For micro-CT analysis, four pedipalps were critically point dried (BAL-TEC 30 critical point drier) after dehydration and mounted on a pin tip with super glue. The X-ray tomography scan was performed with an XRadia Micro XCT-200 (Carl Zeiss X-ray Microscopy Inc.) using the 40x object lens unit, at 40 kV and 4 W, with a pixel size of 0.276 μm . Tomography projections were reconstructed using the reconstruction software provided by XRadia. For image segmentation the software platform Amira 5.4. (FEI, Visualization Science Group) was used. Images from micro-CT scans were compared with semithin sections (0.7 μm) for reciprocal illumination.

PLUG SIZE IN SUCCESSIVE MATINGS

To investigate the potential limitation in the production of the amorphous mating plug in *O. retusus* we conducted consecutive matings of 22 males. Each male mated with three randomly chosen virgin females (total $N = 66$). We amputated one of the paired male pedipalps 3.2 ± 0.7 (SE) days before the mating trials randomly in order to force the male to use the same palp in consecutive matings. Prior to amputation, males were anesthetized with CO_2 . Amputation

does not influence male mating behaviour (Kunz *et al.*, 2012).

Males were on average 4.86 ± 0.36 (SE) days old, first females 8.06 ± 3.29 , second females 3.59 ± 0.33 , and third females 3.32 ± 0.40 , calculated as days after the final molt. Female age between groups was not significantly different (Kruskal–Wallis-test: $N = 66$, Chi-squared = 1.03, d.f. = 2, $P = 0.597$). At the start of the experiment, a male was carefully put into the female's container. We observed male and female courtship and mating behaviours as recorded in Table 1. After copulation (one pedipalp insertion), the male was put back into his own container and observed for 1 h. We recorded the latency to sperm web construction and refilling of pedipalps, i.e. the transfer of sperm produced in the testes to the interim sperm storage organs, the pedipalps, which occurred within the 1 h observation period. Then, the male was transferred into the container of the second female, and experiments were staged the same way as before. The same procedure was applied for the third female.

After the mating trials, all females were transferred into separate vials, anesthetized with CO_2 , and fixed in a Duboscq-Brazil solution (Mulisch & Welsch, 2010). In order to determine the size of the plugs, the genital region was investigated under an SEM (Hitachi S2460N). To this aim, the samples were washed with 80% ethanol, dehydrated in graded series of ethanol, critical point dried (Bal-tec CPD 030), mounted and sputter coated with gold (Polaron SC7620). As a proxy for plug size, we calculated the two-dimensional area of the plug (in μm^2) from SEM photographs of the female genital region using the program Axiovision 4.8. (Carl Zeiss MicroImaging). Female opisthosomata were photographed under a Zeiss Discovery. V20 and their 2D areas were likewise measured and taken as a proxy for fecundity (Uhl, Schmitt & Schäfer, 2004). In order to avoid observer bias, all size measurements (plugs and female opisthosomata) were taken without knowledge of the

Table 1. Courtship, mating behaviour (copulation latency = time interval between start of experiment and copulation; copulation duration = duration of pedipalp insertion) and latency to sperm induction (uptake of sperm from the sperm web into the spermophore) in three successive matings of 22 *Oedothorax retusus* males. Data are given in seconds as means \pm standard deviation. The Friedman test was used to account for repeated measures (d.f. = 2)

	Courtship latency	Copulation latency	Copulation duration	Sperm induction
1st mating	144.6 \pm 159.1	275.2 \pm 204.1	138.5 \pm 59.9	1027.6 \pm 605.5
2nd mating	99.5 \pm 123.9	286.7 \pm 350.5	139.8 \pm 45.4	1142.4 \pm 789.4
3rd mating	127.7 \pm 135.6	228.9 \pm 214.4	124.3 \pm 49.8	1223.7 \pm 691.6
Friedman	$\chi^2 = 1.91, P = 0.385$	$\chi^2 = 0.36, P = 0.834$	$\chi^2 = 0.30, P = 0.861$	$\chi^2 = 3.65, P = 0.161$

identity of the female (blind testing). Statistical analysis was performed using IBM SPSS statistics 21.

RESULTS

PLUG PRODUCTION SITE

The distal part of the male pedipalp is comprised of a cup-shaped cymbium, representing the tarsus of the pedipalp, and the palpal organ, which is connected to the cymbium through a basal membranous area

(basal hematodocha). Inside the complex palpal organ we suspected the site of plug formation. The palpal organ consists of hard, sclerotized parts (sclerites) that extend from the basal hematodocha (Figs 1C, 2A, B). The most distal sclerite represents the actual sperm transferring structure, the embolus that possesses a slightly twisted tip (Fig. 1C). The interim sperm storage site inside the palpal organ, the spermophore, is a spiral duct that opens at the tip of the embolus (Fig. 1D–F) and runs through a number

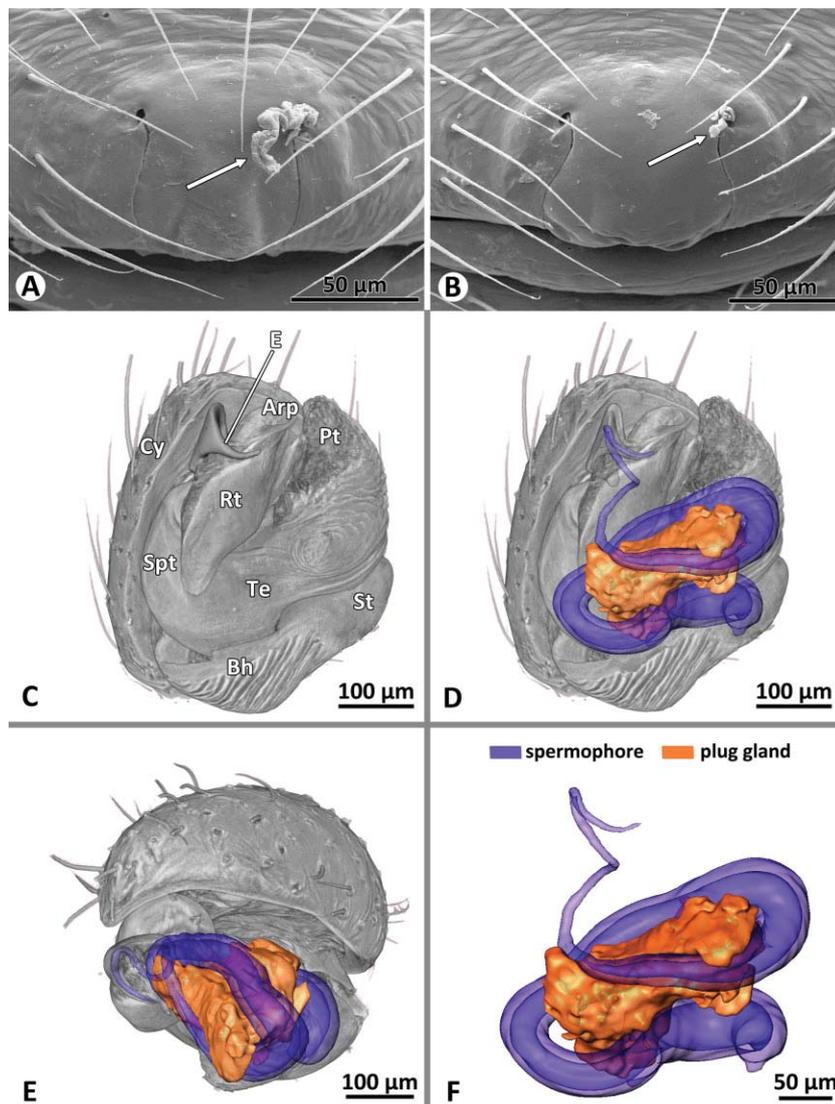


Figure 1. Mating plugs on the female genital openings (A, B) and micro-CT generated 3D-images of surface and selected internal structures of the male pedipalp in *Oedothorax retusus* (C–F). (A) Large amorphous mating plug (arrow) in the left genital opening of a female. (B) Small amorphous mating plug (arrow) in the left genital opening of a female. (C) Volume rendering of the palpal organ. (D–F) Position of the spermophore (blue) and the mating plug producing gland (orange) in different orientations (D, E) and extracted from the surrounding male pedipalp (F). Arp: anterior radical process, Bh: basal hematodocha, Cy: cymbium, E: embolus, Pt: protegulum, Rt: radical tailpiece, Spt: supratégulum, St: subtegulum, Te: tegulum.

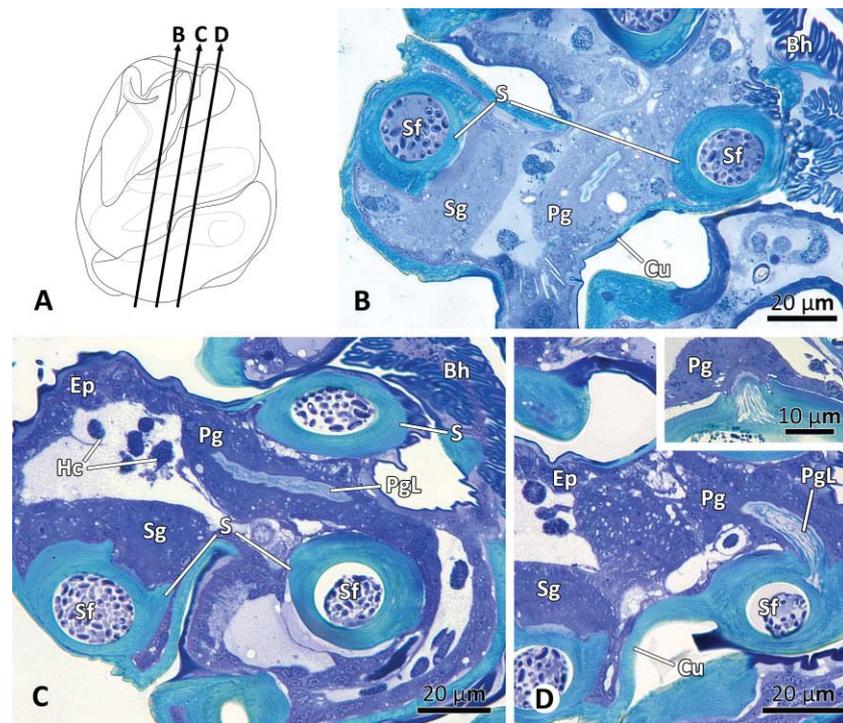


Figure 2. Semithin sections of the palpal organ of *O. retusus* demonstrating the presence of a spermatophore gland (Sg) and a mating plug gland (Pg) that both connect to the spermatophore (S). (A) Schematic drawing of the male palpal organ indicating the position of consecutive sections shown in B–D. (B) The basal hematodocha connects the palpal organ with the cymbium. The spermatophore is curved and runs through several sclerites. (C) The spermatophore gland that partly surrounds the spermatophore, releases its secretion along a distinct rim build of loose cuticle. In contrast, a second gland, the so called ‘mating plug gland’ is located in the centre of the palpal organ. (D) Secretions of the mating plug gland are released into the lumen of the latter near its blind end through an elongated portion of loose cuticle layers that appears striated in cross-sections (inset). Bh: basal hematodocha, Cu: cuticle, Ep: epidermis, Hc: hemocyte, Pg: plug gland, PgL: lumen of the plug gland, S: spermatophore, Sf: seminal fluid, Sg: spermatophore gland.

of sclerites (subtegulum, tegulum, prottegulum) until its blind end. The spiral course of the spermatophore results in several cross sections on one slide (Fig. 2B–D). The spermatophore is accompanied by the spermatophore gland, which runs along a groove in the spermatophore wall (Fig. 2). Another massive glandular tissue is found in the centre of the palpal organ, surrounded by the spermatophore loops (Fig. 1D–F, Fig. 2, see supplementary file in interactive 3D mode). The gland is composed of cells that are densely packed with mitochondria and vesicles (Fig. 3). The vesicles are electron dense, occur in different sizes and are irregularly shaped (Fig. 3A). Close to the passage to the spermatophore, large, electron lucent vesicles with a conspicuous electron dense spot are present (Fig. 3B, inset). Apically, the gland cells exhibit a pronounced microvilli region (Fig. 3A, B). The layers of the adjacent cuticle are loosely connected (Figs 2B–D, 3A) leading to a spacious lumen between the layers. This cuticular structure connects with the spermatophore at approximately one-tenth of

the whole length of the spermatophore measured from its blind end. The spermatophore gland that runs along the spermatophore does not extend to the point where the central gland connects to the spermatophore.

PLUG APPLICATION IN SUCCESSIVE MATINGS

Males did not differ significantly in courtship and copulation latency between successive matings with virgin females (Table 1). Likewise, copulation duration was not significantly different between the three mating trials (Table 1). The time males took to take up sperm after each copulation was also not significantly different between successive trials (Table 1). Overall, copulations were terminated by males with significantly higher probability than by females (termination by male : female (%): first mating: 16 : 6 (72.7%), second mating: 22 : 0 (100.0%), third mating: 19 : 3 (86.4%); Binomial tests: all $P < 0.052$).

In successive matings, the size of the mating plug was not significantly correlated with copulation

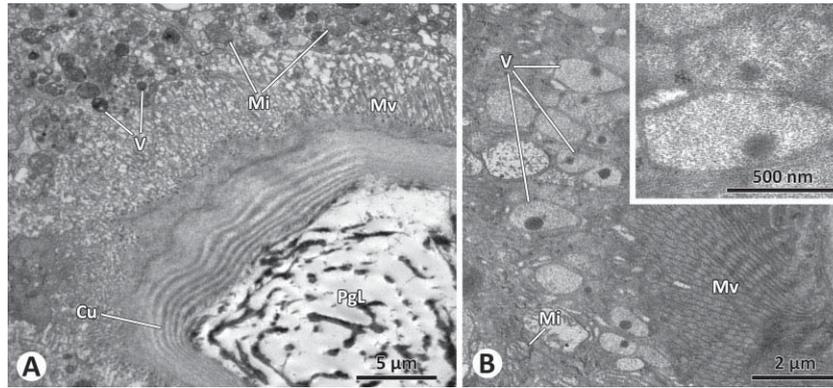


Figure 3. Fine structural details of the mating plug gland in *O. retusus*. (A) Gland cells of the mating plug gland contain numerous mitochondria and electron dense vesicles of different sizes and irregular shapes. Apically, these cells are characterized by a pronounced microvilli zone that surrounds a cuticular layer. (B) Near the passage of the cuticular duct into the spermophore numerous elongated and electron lucent vesicles occur. Cu: cuticle, Mi: mitochondria, Mv: microvilli, PgL: lumen of plug gland, V: vesicle.

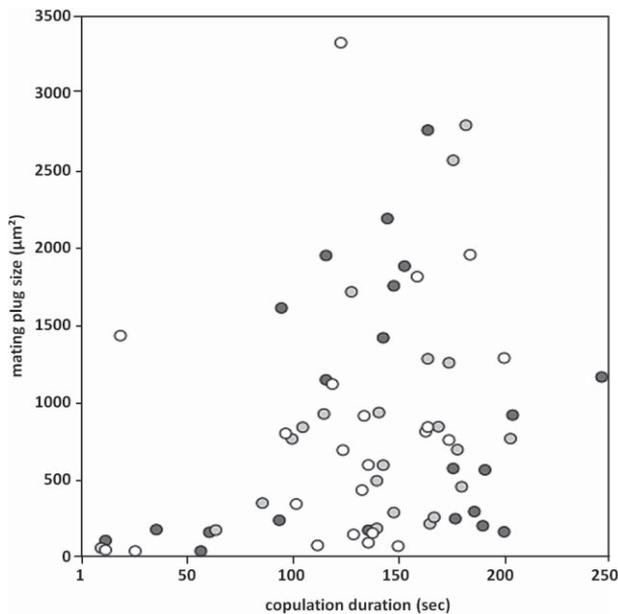


Figure 4. Size of the mating plugs in relation to copulation duration of first (black dots), second (grey dots) and third (white dots) successive matings of 22 male *Oedothorax retusus*.

duration (first mating: $r_s = 0.266$, $P = 0.231$; second mating: $r_s = 0.388$, $P = 0.074$; third mating: $r_s = 0.343$, $P = 0.118$, all $N = 22$) (Fig. 4). Since not all males transferred a large amount of plug material in their first mating, there is no overall decline in plug size with successive matings (plug size: mating 1: $858.01 \pm 175.98 \mu\text{m}^2$ (mean \pm SE), mating 2: $800.63 \pm 154.92 \mu\text{m}^2$, mating 3: $768.39 \pm 170.80 \mu\text{m}^2$; Friedman test: Chi-squared = 0.091, d.f. = 2, $P = 0.956$, $N = 22$) (Fig. 5). However, those males that

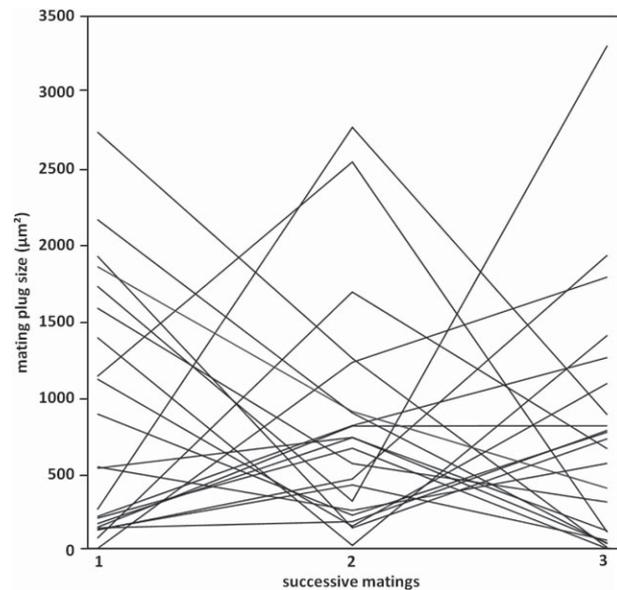


Figure 5. Change in the size of mating plugs produced by 22 male *Oedothorax retusus* over successive matings with three virgin females.

produced a considerable plug mass in the first mating (conservative subset with plug size $> 500 \mu\text{m}^2$) applied a significantly smaller plug in the second mating (Wilcoxon matched pairs test: $U = -2.275$, $P = 0.023$, $N = 12$) and vice versa ($U = -2.805$, $P = 0.005$, $N = 10$). This also applies for second to third matings (both $P < 0.010$) (Fig. 5). Plug size was not significantly related to male age (spearman rank correlation: first plug: $r_s = -0.079$, $P = 0.726$; second plug: $r_s = 0.311$, $P = 0.159$; third plug: $r_s = 0.103$, $P = 0.647$; $N = 22$) nor to female fecundity measured as the two-dimensional area of the female opisthosoma (first mating:

$r_s = 0.132$, $P = 0.559$, second mating: $r_s = 0.019$, $P = 0.934$; third mating: $r_s = 0.399$, $P = 0.066$; $N = 22$).

DISCUSSION

We investigated the plug production site in male *Oedothorax retusus* and whether males are limited in plug material. The palpal organ of the male contains the spermophore, in which the sperm mass is stored until mating, as well as two prominent glands, both connected to the spermophore. We pinpoint the gland that discharges its product close to the blind end of the spermophore as the gland responsible for the production of plug material. Our data indicate that males are limited in the production of plug material.

PLUG PRODUCTION SITE

One of the two glands inside the palpal organ of *O. retusus* runs along the spermophore over more than half of its length, discharging its secretion into the spermophore lumen. This gland seems very similar to the 'spermophore gland' described for two *Amaurobius* species (Suhm *et al.*, 1996). In fact, palpal glands associated with the spermophore are typical for spiders and are suggested to function in the context of uptake and release of the sperm mass or merely in its lubrication (Fickert, 1876; Osterloh, 1922; Harm, 1931; Gerhardt & Kästner, 1937; Cook, 1966; Lopez, 1977; Eberhard & Huber, 2010). If the secretions of the spermophore gland in *O. retusus* were used to seal the females' genital openings, plugs should contain sperm because the secretions and the sperm mass necessarily mix inside the spermophore. However, plugs of *O. retusus* are devoid of sperm (Uhl & Busch, 2009). Therefore, the spermophore gland very likely plays a role in the context of sperm uptake, storage and/or transfer (see references above) but not in the context of monopolizing the female. Therefore, the second gland inside the palpal organ is the obvious candidate for plug production and thus termed 'plug gland'. It is a prominent gland that takes up most of the space in the center of the palpal organ and is encircled by the spermophore loops. The gland cells contain numerous vesicles and possess a marked microvilli region apically, which typically occurs in epidermal gland cells (e.g. Noirot & Quennedey 1974 for insects). The glandular units are devoid of glandular ducts suggesting that they are single layered and unicellular. Like the spermophore gland, the plug gland discharges its secretion into the spermophore, however, shortly before its blind end. Consequently, the ejaculate and the plug secretion have to be transferred through the same exit with the plug mass being transferred last. Such consecutive transfer corresponds with the finding that plugs are

devoid of sperm after mating with virgin females (Uhl & Busch, 2009). Interestingly, this also holds for short matings of only one minute that were terminated experimentally (Uhl & Busch, 2009; Kunz *et al.*, 2014), suggesting that sperm transfer occurs rapidly and transfer of plug material starts already within the first minute of mating. Since copulations lasted around 2 min in our study (Table 1), the transfer of plug material seems to be more time consuming than sperm transfer. A decreasing rate of transfer of material in the course of mating may arise from the declining rate of hematochoal inflations of the palp during copulation as was observed in *O. retusus* (Kunz K, personal observation). Similarly, the frequency of palpal movements in the pholcid spider *Pholcus phalangioides* considerably decline in the course of copulation (Schäfer & Uhl, 2002). In the latter species, the frequency of movements is a better predictor of the number of spermatozoa transferred than copulation duration, although both parameters are highly correlated (Uhl G & Gabel E, unpublished). The hydraulic mechanism that underlies spider locomotion in general also determines the movability of palpal sclerites and causes the rhythmic inflations of the hematochoae (Huber, 2004; Foelix, 2011). Hydraulic insufficiency, as well as biochemical limitation, i.e. the accumulation of anaerobic by-products in phases of high activity, is suspected to be responsible for the early fatigue that characterizes spider movement (Anderson & Prestwich, 1975; Prestwich, 1988). Early fatigue thus seems to strongly constrain the speed of ejection of material from the palp, be it sperm mass or plug material. Therefore, species that are restricted to consecutive transfer of sperm and plug mass require more time compared with species that can transfer both masses at the same time. In the amaurobid spiders *Amaurobius fenestralis* and *A. ferox* the plug gland possesses a separate opening at the base of the embolus (Suhm *et al.*, 1996). Accordingly, males are able to place large plugs despite very short matings. Rapid transfer of sperm mass via the embolus tip into the spermathecae and plug mass via the separate plug opening onto the genital atrium must occur at the same time in these species. Consequently, the plug mass contains some spermatozoa close to the copulatory ducts whereas most of the plug material consist of the heterogeneous material found inside the bulbus gland (Suhm *et al.*, 1996). At least theoretically, a separate opening for plug material allows males to place a plug as soon as the female attempts to end the mating prematurely. Male *O. retusus*, on the other hand, are expected to be constrained in their ability to react to female decisions since they can only apply plug material after sperm transfer. Possibly, male *O. retusus* transfer a relatively small amount of sperm sufficient for

fertilizing the 20–30 eggs that are laid during an oviposition event (Kunz *et al.*, 2012), leading to a fast transfer of ejaculate and time for the application of the mating plug. In fact, surprisingly small amounts of spermatozoa were found inside the male palp and inside the female genital tract (Uhl G & Gabel E, unpublished). Males may need to trade-off sperm and plug mass as suggested in a mathematical model exploring the evolution of mating plugs (Fromhage, 2012). However, in order to understand the constraints involved in transfer of sperm and plug material a detailed biophysical analysis of the underlying hydraulic processes is required.

PLUG SIZE, LIMITATION AND POTENTIAL MATE CHOICE

The size of the mating plug in *O. retusus* was related to copulation duration, however, not significantly so for all matings of a male. In previous experiments, in which categories of copulation duration were used (1 min or 3 min copulation duration) plug size was significantly smaller in the short copulation duration category (Uhl & Busch, 2009; Kunz *et al.*, 2014). Our present study shows that males did not consistently produce a large plug during their first mating and smaller ones in the following matings. Plug sizes were highly variable already in the first mating, suggesting either that the males' ability in producing plug material differed, that males economized on plug material differentially or that plug application can be controlled by the female. Male age did not cause the variability in plug size. In case the amount of plug mass applied is decided on by the male, males may hold back plug material if they can perform male mate choice during mating, and if further mating opportunities are likely. However, in our study plug size was not related to an indicator of female fecundity. The amount of plug material transferred may also depend on the probability of finding another receptive female or the risk of female remating with another male. Fine-tuned male mating responses to the socio-sexual situation have already been reported from insects and spiders (e.g. Gage & Baker, 1991; Simmons, 2001; Schaefer & Uhl, 2003). Nevertheless, economizing on plug material would lead to lost investment in ejaculate and time since the female may mate with another male. In species with sequential rather than simultaneous mate encounters and low mate availability, male mate choice does not easily evolve even in species with large variation in female fitness prospects and high mating costs for males (Barry & Kokko, 2010). Since genital plugging results in low availability of receptive females, at least theoretically males should take advantage of every mating opportunity and secure paternity by applying sufficient amounts of plug material. Alter-

natively, females may determine how much material a given male is able to transfer and thus how safely he can monopolize access to her copulatory opening. In some species, females differentially contribute material to the mating plug thereby deciding on whether the male will father her offspring exclusively or not (Aisenberg & Eberhard, 2009). So far, however, we have not found any indications of female selective cooperation in plug production in *O. retusus* (Kunz *et al.*, 2014) but it seems possible that females can selectively limit plug applications by males. Although our data set cannot fully explain the high variability in plug sizes, it nevertheless suggests that males are restricted in the production of plug material since the application of large plugs in the first mating resulted in a high probability of smaller plugs in the second mating and vice versa.

In conclusion, our study demonstrates that the plug material in *O. retusus* is produced by a gland that is located inside the palpal organ along with the spermophore gland. The plug gland discharges its product into the spermophore close to its blind end, resulting in consecutive transfer of sperm and plug mass. Our data suggest that males are limited in the production of plug mass. Considering the widespread occurrence of amorphous mating plugs in the animal kingdom, the possibility of male limitation in plug production requires wider attention since it constrains not only male paternity success but also cryptic female choice in species in which females test the males' ability in protecting sperm.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. 3D reconstruction of the male spermophore and plug gland inside the male palp of *Oedothorax retusus*. The interactive 3D-mode can be activated by clicking on the image, allowing the user to rotate, move and magnify the model, to isolate elements and to change the light settings.

Do the size and age of mating plugs alter their efficacy in protecting paternity?

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Do the size and age of mating plugs alter their efficacy in protecting paternity?

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Abstract An obvious means to secure paternity is the production of a mating plug that blocks the female genital opening after mating. Although the mechanical efficacy and persistence of plugs on/in the female genital openings are key traits that determine the degree of paternity protection, these factors have hardly been explored. We therefore investigated the influence of the size of the amorphous plug material (experimentally terminated mating duration as a proxy) and age of the mating plug (time interval between copulations with two successive males) on the efficacy of the plug by analysing the mating success of subsequent males in the dwarf spider *Oedothorax retusus* (Linyphiidae: Erigoninae). Overall, subsequent males attempted to mate in 82 % of trials but only 32.5 % of these resulted in copulation, demonstrating that the plugs are effective safeguards against remating. Remating probability was significantly higher after previous short copulations (~small plug size) compared to long copulations (~large plug size). In the small plug group, fresh plugs (short remating intervals) were significantly less effective compared to older plugs. In the large plug group, remating probability was similarly low over all remating intervals. The observed copulations, however, do not necessarily result in sperm transfer, since sperm masses were found on the plugged female genital area. Our study on *O. retusus* shows that mating plugs are a powerful mechanical safeguard whose efficacy varies with plug size and age. We discuss these findings in the light

of theoretical considerations on the evolution of effective mating plugs.

Keywords Mating plug · Sexual selection · Sperm competition · Monopolization · Paternity protection · Dwarf spider · Erigoninae

Introduction

In polyandrous species, post-copulatory male-male competition occurs if sperm of different males are stored within the female genital tract (Parker 1970). Polyandry is widespread in various animal taxa and has led to numerous behavioural, physiological and morphological male adaptations against sperm competition (Birkhead and Møller 1998; Parker 1998; Simmons 2001; Eberhard 2009). Adaptations can be offensive by reducing mating success of previous males or defensive by impeding or reducing further matings of the female (Parker 1970). Offensive adaptations are, for example, when rival sperm are scraped out of the female genital tract (Simmons 2001), displaced and/or replaced (e.g. Gack and Peschke 1994) or sealed off with a hardening ejaculate (Diesel 1990). A classical defensive male adaptation against multiple female mating is the production of a mating plug. Mating plugs are structures that mechanically block female genital openings after mating and reduce female remating probabilities or impede further matings, thereby securing paternity of the plug-producing male (Simmons 2001). Mating plugs are known from virtually all animal groups (Birkhead and Møller 1998). However, material on or in the female genital openings is often assumed to function as a mating plug without investigating whether it actually limits remating probability of the female. The materials may serve other functions than paternity protection such as preventing sperm leakage and may even be produced by the female (e.g. Aisenberg and Barrantes 2011).

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Moreover, even if the materials were demonstrated to serve as paternity protection devices, the conditions and constraints that shape their efficacy are still largely underexplored.

Supposed mating plugs were found in many spider families (Austad 1984; Uhl et al. 2010). The genital morphology of entelegyne spiders seems to promote the occurrence of mating plugs since females possess three genital openings—two are used for copulation and one for oviposition (Uhl et al. 2010). This allows the male to obstruct the copulatory ducts without impeding oviposition. Further, spiders possess a high potential for sperm competition since females frequently mate with several males, and sperm can be stored over long periods of time in the female genital tract (Elgar 1998). Supposed mating plugs in spiders consist primarily of amorphous material but can also consist of parts of or complete male pedipalps, the secondary copulatory organs (Uhl et al. 2010). Recently, genital fragments were shown to be effective plugs in the orb-weaver *Argiope keyserlingi* (Herberstein et al. 2012). However, in many species, several male fragments can be found in the female genital system, demonstrating that subsequent matings are possible (Uhl et al. 2010).

Here, we focus on amorphous materials on/in the female genital opening since this type of potential mating plug is particularly widespread in the animal kingdom including spiders (Uhl et al. 2010). When the material is transferred from the male to the female, it must be transferred in a liquid state and has to solidify to a certain degree if it is meant to withstand penetration or removal by a rival male. We therefore hypothesize that plugs are not as effective immediately after their placement compared to after curing. Since a plug only helps to secure paternity if it remains functional until oviposition, the degree of persistence of the material is a further crucial aspect that determines paternity success. Furthermore, plug efficacy may strongly depend on plug size if large amounts of material are more difficult to remove compared to small amounts (e.g. Masumoto 1993; Polak et al. 2001). In the butterfly *Cressida cressida*, large mating plugs even present lifelong chastity belts (Orr and Rutowski 1991).

In this study, we follow up on findings in the dwarf spider *Oedothorax retusus* (Linyphiidae: Erigoninae) in which an amorphous mass is found on the female copulatory opening after mating (Uhl and Busch 2009). Mating plugs are significantly larger after longer matings which allows using copulation duration as a proxy for plug size (Uhl and Busch 2009). When experimentally terminating matings after 1 or 3 min, small mating plugs proved to be ineffective but large plugs prevented remating of the female in 93 % of cases. In this previous study, all remating trials were staged within 15 min. However, in order to understand the full potential and limitations of mating plugs, we need to assess how plug size, plug age and their interaction determine plug efficacy.

We hypothesize that mating plug efficacy in *O. retusus* depends (i) on first mating duration (short, long) which can

be used as a proxy for plug size and (ii) on plug age taken as the time interval between first copulation and the second mating trial (15 min; 1 h; 1 day; 3 days after mating; after oviposition). Since cleaning behaviour by the female may potentially alter plug presence and size, we observed if females cleaned the genital region during 1 h post-mating. When subsequent males attempted or succeeded in remating, we investigated the condition of the mating plugs under the SEM. In order to test if female receptivity and attractiveness to males may depend on plug size and age, we further registered aggressive behaviour by females towards males and male courtship probability.

Material and methods

Study species: *O. retusus*

The dwarf spider *O. retusus* belongs to the Erigoninae (Miller and Hormiga 2004) which is the largest group within the Linyphiidae. We used males and females that were raised in the laboratory from egg sacs of females caught along the banks of the river Rhine south of Bonn, Germany (50° 43' 00.26" N, 7° 08' 25.11" E–50° 42' 57.40" N, 7° 08' 36.70" E). Adult spiders were kept individually in 25 ml plastic containers equipped with a moist layer of gypsum at a temperature of 23/17 °C (day/night) and 70 % humidity. They were fed weekly with four to six fruit flies, *Drosophila melanogaster*. Spiderlings were fed *ad libitum* on springtails, *Sinella curviseta*, until the subadult stage (Kunz et al. 2012).

Courtship and copulation in *O. retusus*

Courtship in *O. retusus* starts with the male vibrating his opisthosoma. As soon as the male achieves the contralateral mating position, he stretches one of his pedipalps (secondary copulatory organs) towards the external female genital structure (epigyne) and tries to insert into one of two female copulatory openings. Copulation starts when the male successfully inserts a pedipalp into the corresponding female copulatory opening and the hematodocha (membranous part of the sperm transfer organ) inflates. The insertion mode is ipsilateral, meaning that the right pedipalp is inserted into the right female copulatory opening, which is typical for entelegyne spiders (Huber and Senglet 1997). A mating plug in *O. retusus* appears as a transparent mass shortly after copulation that transforms into solid opaque material (Kunz and Witthuhn, personal observations).

Influence of plug size and age on paternity protection

We investigated plug efficacy of *O. retusus* by staging remating trials that varied in plug size as determined by first male copulation durations (Uhl and Busch 2009) and plug age

as determined by latencies between first and second matings. To make sure that both first and second males used the same of the two female genital openings, one of the two pedipalps of each male was amputated on the same side. As a consequence, both males of a mating trial were restricted to using either the right or the left pedipalp.

Courtship and mating behaviours of males and females were observed as described in Kunz et al. (2012). The observation period started when the first male was placed in the container of a virgin female and ended with copulation or after 1 h. Copulations were terminated by the experimenter after 1 or 3 min in order to create the two treatment groups with a high probability of small (1 min) and large (3 min) mating plugs (Uhl and Busch 2009). Undisturbed copulations with one pedipalp last on average 3.21 min in *O. retusus* (Richter 2006). In the following, we use the terms “small plug group” for those remating trials in which the female previously had experienced a 1-min copulation and “large plug group” for those trials in which the female had experienced a 3-min copulation. Subsequent mating trials were staged after five different intervals: 15 min, 1 h, 1 day, 3 days after mating, or after the female had oviposited (same day or day after). These intervals serve as categories for plug age. When copulations occurred in remating trials, they were not terminated by the experimenter. Second males were removed after copulation or after 1 h of observation time. In 19.9 % of cases (38 out of 191), second males did not try to assume mating position and were replaced by third males. In the following, second or third males whose pedipalps were in contact with the female genital area are treated as “second mating partners”. All males were used for a single mating only.

Of all staged mating trials, 45.4 % (191 out of 421) were used for the analysis (total $N=99$ for the 1-min group: 15 min $N=20$; 1 h $N=19$; 1 day $N=20$; 3 days $N=20$; oviposition $N=20$; and total $N=92$ for the 3-min group: 15 min $N=19$; 1 h $N=17$; 1 day $N=19$; 3 days $N=20$; oviposition $N=17$). During mating trials, we recorded aggressive behaviour of females towards males (attacking and chasing away of the potential mate, Kunz et al. 2012) to detect potential effects of plug size and age on female receptivity. Further, females were observed for 1 h after their first mating in order to detect potential cleaning activity of the genital region that may influence the presence and state of the mating plug. Females of short remating intervals were observed for cleaning activity during their second mating trial.

We controlled for male and female age calculated as days elapsed from the final moult. Females of the small plug group were on average 26.4 ± 4.6 days, and those of the large plug group were on average 25.9 ± 3.5 days old (Mann-Whitney U test: $N=99$, $Z=-0.01$, $p=0.361$). Males of the small plug group were 26.4 ± 4.5 days old, and males from the large plug group 26.0 ± 3.8 days old (Mann-Whitney U test: $N=92$, $Z=-0.89$, $p=0.376$).

Scanning electron microscopy

Female opisthosomata ($N=143$) of those trials in which remating attempts or rematings occurred were fixed in Dubosq-Brazil solution (Mulisch and Welsch 2010) in order to investigate the condition of the mating plug. Seventy females were investigated from the small plug group (15 min $N=12$; 1 h $N=14$; 1 day $N=18$; 3 days $N=14$; oviposition $N=12$), and 73 females from the large plug group (15 min $N=15$; 1 h $N=12$; 1 day $N=17$; 3 days $N=15$; oviposition $N=14$). After a minimum of 2 days of infiltration, the samples were dehydrated in an ascending ethanol series, chemically dehydrated with 1,1,1,3,3,3-hexamethyldisilazane (HMDS), sputter-coated for 200 s with gold/palladium (80/20) (Polaron SC7640, Fisons Instruments) and investigated under a SEM EVO LS10 (Zeiss) at the Imaging Centre of the University of Greifswald. We inspected the female genitalia for presence, location (right/left copulatory duct), size category (small/large) and whether spermatozoa were visible in the plug material.

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics version 20. Normally distributed data are given as arithmetic mean \pm standard deviation (mean \pm SD). Non-normally distributed data are given as median, interquartile range (IQR), and are ln-transformed for GLM analysis. GLM analyses were performed with the parameters “plug size” (1-min group and 3-min group), “plug age” (15 min, 1 h, 1 day, 3 days, oviposition) and “interaction between size and age” and are given as Wald χ^2 or likelihood ratio χ^2 (LR χ^2) in the case of transformed data. For binomial data, we used GLM with binomial distribution and logit link function. None of the interactions were significant (all $p>0.178$). We further explored significant parameters from the GLM analysis using χ^2 and Mann-Whitney U test.

Results

Mating plugs as a mechanical safeguard?

We analysed if mating plug efficacy depends on plug size (1 and 3 min during first copulation) and/or plug age (time interval between first and second copulation) by investigating copulation attempts and remating success. One hundred fifty-seven out of 191 (82.2 %) second mating partners performed copulation attempts with their single pedipalp on the plugged female copulatory opening. The probability of copulation attempts was not different between the plug size groups (GLM: copulation attempts yes/no: small plug group, 78/21 (78.8 %); large

plug group, 79/13 (85.9 %); Wald $\chi^2=0.00$, $df=1$, $p=0.999$) but significantly different between plug age classes (copulation attempts yes/no: 15 min, 36/3 (92.3 %); 1 h, 35/1 (97.2 %); 1 day, 34/5 (87.2 %); 3 days, 33/7 (82.5 %); oviposition, 19/18 (51.4 %); Wald $\chi^2=13.72$, $df=4$, $p=0.008$). The lower probability of copulation attempts in the oviposition group accounted for this significant difference. When excluding the oviposition interval, there was no significant effect between the remaining plug age classes (Wald $\chi^2=0.19$, $df=3$, $p=0.980$) (see Table 1 for data per size and age groups). The number of copulation attempts a male performed was marginally significantly different between plug size groups (GLM: number of copulation attempts: small plug group ($N=78$) 16, IQR 41; large plug group ($N=79$) 24, IQR 57; LR $\chi^2=3.51$, $df=1$, $p=0.061$), whereas the number of copulation attempts differed significantly between plug age classes (number of copulation attempts: 15 min ($N=36$) 17, IQR 42; 1 h ($N=35$) 24, IQR 85; 1 day ($N=34$) 24, IQR 49; 3 days ($N=33$) 24, IQR 65; oviposition ($N=19$) 6, IQR 19; LR $\chi^2=12.27$, $df=4$, $p=0.015$). The significant effect can be attributed to the low number of copulation attempts in the oviposition group. When removed from the analysis, the number of copulation attempts was not significantly different between plug age classes (LR $\chi^2=4.09$, $df=3$, $p=0.252$) (see Table 1 for data per size and age groups).

Altogether 32.5 % of males (62 out of 191) copulated with a mated female. The overall probability of remating was significantly higher for the small plug group (GLM: remating yes/no: small plug group, 45/54 (45.5 %); large plug group, 17/75 (18.5 %); Wald $\chi^2=14.39$, $df=1$, $p<0.001$). Likewise, plug age classes overall revealed significantly different female remating probabilities (remating yes/no: 15 min, 22/17 (56.4 %); 1 h, 15/21 (41.7 %); 1 day, 13/26 (33.3 %); 3 days, 7/33 (17.5 %); oviposition, 5/32 (13.5 %); Wald $\chi^2=17.17$, $df=4$, $p=0.002$). In the small plug group, plug age class had a highly significant effect on remating probabilities (Table 1; Fig. 1, light grey bars). Only the 15-min age class accounted for the significant effect since the other age classes (1 h to after oviposition) did not differ significantly when compared without the 15-min age class ($\chi^2=6.53$, $df=3$, $p=0.088$). Differences between plug size groups remained significant after excluding the 15-min interval from the analysis (GLM: remating yes/no: small plug group, 28/51 (35.4 %); large plug group, 12/61 (16.4 %); Wald $\chi^2=7.18$, $df=1$, $p=0.007$). In the large plug group, overall remating probabilities were low and not significantly different between plug age classes (Table 1; Fig. 1, dark grey bars).

The copulation duration of subsequent males was not significantly different between the plug size groups (GLM: copulation duration in seconds: small plug group ($N=45$), 154.8 ± 96.2 ; large plug group ($N=17$), 126.5 ± 103.4 ; Wald $\chi^2=1.52$,

Table 1 Courtship and mating parameters of *Oedothorax retusus* in mating trials with females bearing small plugs or large plugs. Data are given for five plug age classes (intervals between first and second copulation: 15 min, 1 h, 1 day, 3 days, after oviposition). Data are given as events (proportions), mean \pm standard deviation, and median, interquartile range (IQR). Statistical analysis was performed using χ^2 test, ANOVAs (F) and Kruskal-Wallis tests. Significant p -values are shown in bold. Overall GLM results for the parameters plug size and plug age plus interaction are given in the text

	Plug	15 min		1 h		1 day		3 days		Oviposition		F^a, χ^2	p
		N	Mean/median	N	Mean/median	N	Mean/median	N	Mean/median	Events	Mean/median		
Copulation attempts (yes/no)	Small	20	17/3 (85.0 %)	19	18/1 (94.7 %)	20	18/2 (90.0 %)	20	15/5 (75.0 %)	20	10/10 (50.0 %)	14.95	0.005
	Large	19	19/0 (100.0 %)	17	17/0 (100.0 %)	19	16/3 (84.2 %)	20	18/2 (90.0 %)	17	9/8 (52.9 %)	21.44	<0.001
Copulation attempts [no.]	Small	17	10, IQR 27	18	14, IQR 33	18	32, IQR 90	15	17, IQR 60	10	11, IQR 20	5.55 ^b	0.235
	Large	19	36.4 \pm 35.1	17	69.8 \pm 65.6	16	34.3 \pm 32.9	18	45.2 \pm 43.9	9	12.2 \pm 12.1	3.00 ^a	0.024
Remating (yes/no)	Small	20	17/3 (85.0 %)	19	11/8 (57.9 %)	20	7/13 (35.0 %)	20	6/14 (30.0 %)	20	4/16 (20.0 %)	21.84	<0.001
	Large	19	5/14 (26.3 %)	17	4/13 (23.5 %)	19	6/13 (31.6 %)	20	1/19 (5.0 %)	17	1/16 (5.9 %)	7.43	0.115
Copulation duration [s]	Small	17	156.6 \pm 95.6	11	164.0 \pm 72.1	7	130.6 \pm 119.9	6	154.2 \pm 117.2	4	165.3 \pm 126.8	0.14 ^a	0.967
	Large	5	161.2 \pm 138.6	4	110.0 \pm 93.4	6	123.3 \pm 98.5	1	147.0 \pm 0.0	1	17.0 \pm 0.0	0.39 ^a	0.813

^a ANOVAs (F)

^b Kruskal-Wallis tests

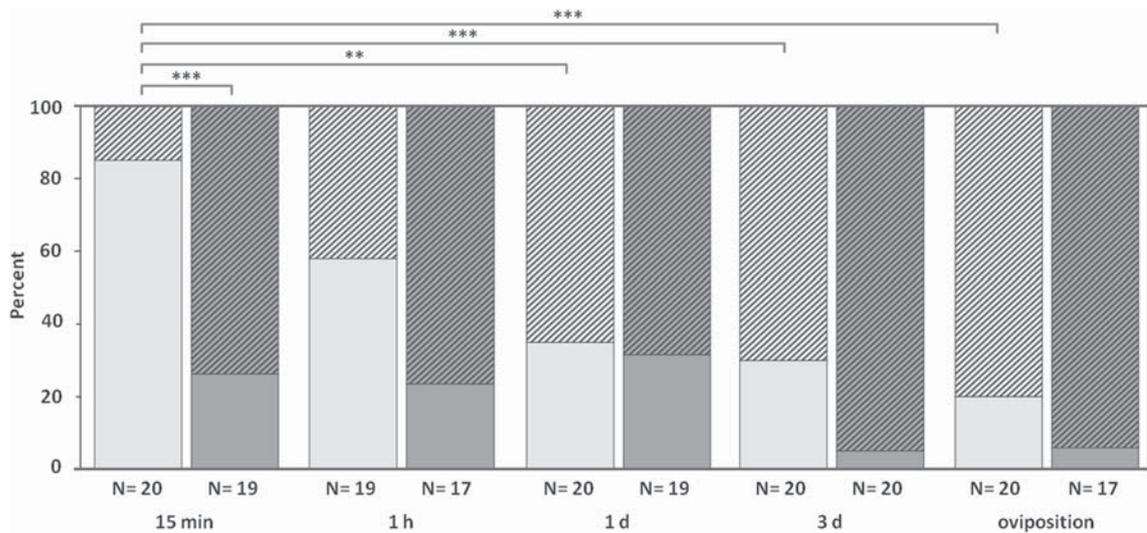


Fig. 1 Percentages of copulations (full bars) and unsuccessful copulation attempts (striped bars) in *Oedothorax retusus* mating trials with mated females that bear small (light grey bars) or large mating plugs (dark grey bars). Data are given for five plug age classes (interval between first

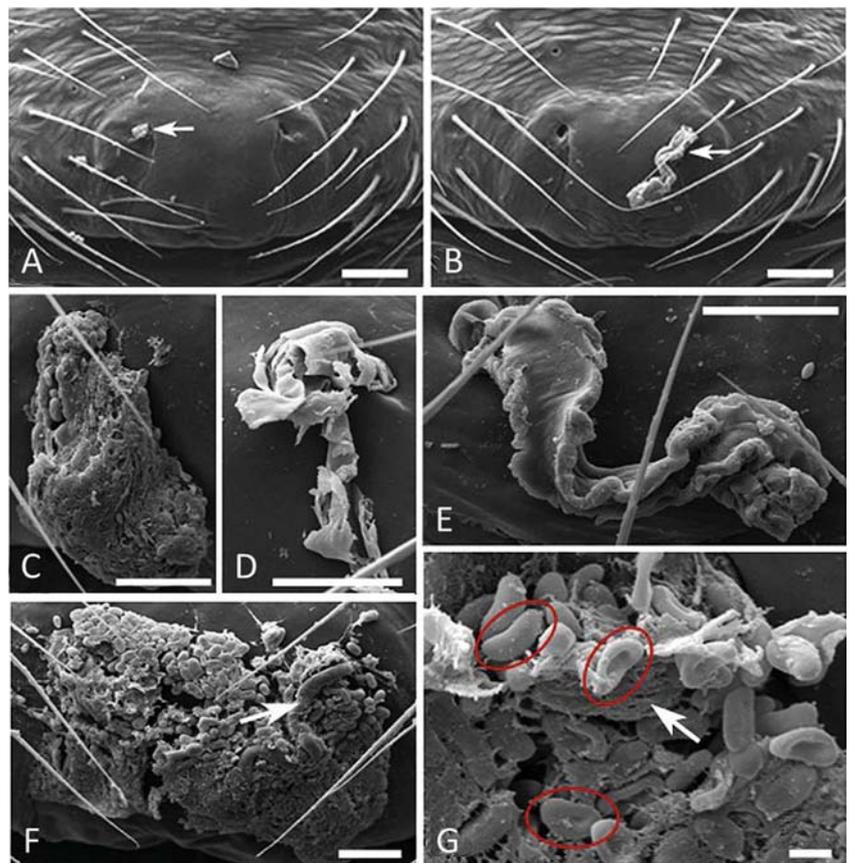
mating and second mating trial: 15 min, 1 h, 1 day, 3 days, after oviposition). Asterisks mark significant differences in pairwise χ^2 tests after sequential Bonferroni correction (** $0.001 \leq \alpha \leq 0.01$, *** $\alpha < 0.001$)

$df=1$, $p=0.218$) nor between plug age classes (15 min ($N=22$), 157.6 ± 103.1 ; 1 h ($N=15$), 149.6 ± 78.7 ; 1 day ($N=13$), 127.2 ± 106.1 ; 3 days ($N=7$), 153.1 ± 107.0 ; oviposition ($N=5$), 135.6 ± 128.3 ; Wald $\chi^2=1.78$, $df=4$, $p=0.777$) (see Table 1 for data per size groups and age classes).

State of the mating plug after remating

In those cases in which remating occurred, the plug material contained spermatozoa (Fig. 2) with significantly higher probability than in cases in which only copulation attempts were

Fig. 2 SEM photographs of the genital regions of mated *Oedothorax retusus* females. **a** Small mating plug (arrowhead) in the right side of the female genital opening typical for the 1-min group (once mated female). **b** Large mating plug (arrowhead) in the left side of the female genital opening typical for the 3-min group (once mated female). **c** Large, inhomogeneous mating plug (once mated female). **d** Damaged mating plug (second male performed 67 copulation attempts). **e** Large, loopy, homogenous mating plug (once mated female). **f** Plane, previously loopy (arrowhead) mating plug including spermatozoa from a subsequent mating. **g** Spermatozoa ($\sim 3 \mu\text{m}$, red circles) and amorphous secretion (arrowhead) after remating. Scale bars: **a–f** $20 \mu\text{m}$, **g** $2 \mu\text{m}$



observed (sperm yes/no: successful remating, 27/11 (71.1 %); unsuccessful remating, 14/91 (13.3 %); $\chi^2=45.45$, $df=1$, $p<0.001$). This result holds for the small plug group where most rematings occurred (sperm yes/no: successful remating, 21/8 (72.4 %); unsuccessful remating, 3/38 (7.3 %); Fisher's exact test: $p<0.001$) as well as for the large plug group (sperm yes/no: successful remating, 6/3 (66.7 %); unsuccessful remating, 11/53 (17.2 %); Fisher's exact test: $p=0.004$).

The SEM analysis of the state of the mating plug after remating revealed that 58 % of mating plugs show areas of broken off plug material (sharp edges) (Fig. 2d) that were not observed in previous investigations on once mated females (Uhl and Busch 2009).

Potential influence of plug size and age on female attractiveness and receptivity

To assess the potential effects of size and age of the mating plug on female attractiveness, we analysed courtship behaviour of second mating partners. Courtship probabilities of second males were not different between plug size groups (GLM: courtship yes/no: small plug group, 97/2; large plug group, 92/0; Wald $\chi^2=0.00$, $df=1$, $p=0.999$). There was also no difference in courtship probability between plug age classes (courtship yes/no: 15 min, 39/0; 1 h, 36/0; 1 day, 39/0; 3 days, 39/1; oviposition, 36/1; Wald $\chi^2=0.00$, $df=4$, $p=1.000$). Courtship latency of second males was also not significantly different between the plug size groups (GLM: courtship latency in seconds: small plug group ($N=97$) 124.4 ± 316.6 ; large plug group ($N=92$) 147.6 ± 385.1 ; Wald $\chi^2=0.20$, $df=1$, $p=0.653$) nor between plug age classes (15 min ($N=39$), 115.1 ± 202.7 ; 1 h ($N=36$), 120.6 ± 234.8 ; 1 day ($N=39$), 159.4 ± 433.1 ; 3 days ($N=39$), 216.7 ± 543.6 ; oviposition ($N=36$), 59.8 ± 149.3 ; Wald $\chi^2=4.22$, $df=4$, $p=0.377$). Likewise, the time it took to achieve mating position was not significantly different between the plug size groups (GLM: latency mating position: small plug group ($N=83$) 254, IQR 443; large plug group ($N=81$) 273, IQR 725; LR $\chi^2=0.72$, $df=1$, $p=0.398$) nor between plug age classes (latency mating position: 15 min ($N=39$) 302, IQR 746; 1 h ($N=36$) 239, IQR 537; 1 day ($N=36$) 220, IQR 352; 3 days ($N=33$) 274, IQR 505; oviposition ($N=20$) 399, IQR 1,087; LR $\chi^2=5.51$, $df=4$, $p=0.239$).

We further analysed aggressive behaviour of females towards second mating partners in order to assess the potential effects of mating plug size and age on female receptivity. The probability of female aggressiveness was not significantly different in remating trials between plug size groups (GLM: female aggressiveness yes/no: small plug group, 24/75 (24.3 %); large plug group, 14/78 (15.2 %); Wald $\chi^2=1.48$, $df=1$, $p=0.224$) nor between plug age classes (female aggressiveness yes/no: 15 min, 3/36 (7.7 %); 1 h, 4/32 (11.1 %); 1 day, 10/29 (25.6 %); 3 days,

8/32 (20.0 %); oviposition, 13/24 (35.1 %); Wald $\chi^2=8.16$, $df=4$, $p=0.086$). The number of aggressive interactions was not significantly affected by plug size (GLM: number of aggressive interactions: small plug group ($N=24$) 1, IQR 2; large plug group ($N=14$) 1, IQR 2; LR $\chi^2=0.31$, $df=1$, $p=0.579$) nor by plug age (number of aggressive interactions: 15 min ($N=3$) 0, IQR 0; 1 h ($N=4$) 3, IQR 5; 1 day ($N=10$) 1, IQR 0; 3 days ($N=8$) 1, IQR 1; oviposition ($N=13$) 2, IQR 4; LR $\chi^2=6.94$, $df=1$, $p=0.139$).

Can female cleaning behaviour affect plug presence and size?

Female post-copulatory cleaning behaviour may influence plug presence and size. Cleaning after copulation occurred in 6.3 % of females (12 out of 191). Our experimental plug size groups did not significantly differ in cleaning probability (cleaning yes/no: small plug group, 6/93 (6.1 %); large plug group, 6/86 (6.5 %); $\chi^2=0.02$, $df=1$, $p=0.896$). Due to the rare occurrence of cleaning behaviour, plug age classes were not analysed.

Discussion

Polyandrous mating combined with long-term sperm storage is particularly widespread in insects and spiders (Elgar 1998; Simmons and Siva-Jothy 1998), and mechanical safeguards against polyandrous mating are suspected to have evolved many times independently. The mating plug in *O. retusus* clearly functions as a mechanical obstacle to rival males. Overall, small plugs are less effective than large plugs. Shortly after plug placement, small plugs are least effective suggesting that size and hardening of the material plays an important role. Once plugs are older than a day, they strongly serve to monopolize access to the duct that leads to the female sperm storage organ. However, even if subsequent males are able to mate, at least part of their sperm mass remains outside of the female genital tract, which considerably adds to the efficacy of the mating plug. Consequently, if a male succeeds in plugging both of the females' copulatory openings, his paternity success is expected to be very high.

There is increasing evidence that mating plugs can be produced by the female (Aisenberg and Eberhard 2009; Aisenberg and Barrantes 2011; Kuntner et al. 2012), demonstrating that detailed analysis of the origin of the substance is a basic requirement to understand the adaptive context in which the plug evolved. In our model species *O. retusus*, the mating plug is produced inside of the male copulatory organs, the pedipalps (Uhl et al. 2014) and there is no indication that females participate in plug production or can remove the plug after mating. As shown here, the efficacy of the mating plug is strongly shaped by plug size,

with efficacy greater in large plugs. This suggests that smaller portions of plug material can be more easily removed or overcome by a subsequent male as was also shown for some insects and spiders (Jackson 1980; Matsumoto and Suzuki 1992; Masumoto 1993). Plug age can also determine plug efficacy, although this effect occurred only for small plugs, which became more effective as they aged, suggesting that hardening of the material is required (see also Matsumoto and Suzuki 1992).

It is not clear whether males that copulated with mated females were able to transfer sperm, since sperm were found on the genital area, suggesting that the paternity protection afforded by mating plugs is even greater than that suggested by remating probabilities. Instead of operating as a physical barrier, mating plugs may alter female behaviour and receptivity (Eberhard 2004; Colonello and Hartfelder 2005; Wedell 2005; Avila et al. 2011) or reduce female attractiveness for subsequent males (Parker 1970; Shine et al. 2000; O'Donnell et al. 2004). In *O. retusus*, we cannot disentangle potential effects of mating from those of the mating plug itself, since mating results in a visible plug in 98 % of cases (Uhl and Busch 2009). However, the level of female receptivity did not depend on plug size and age. Only after oviposition, female aggressiveness towards the male was increased, possibly due to a short phase of brood care (Kunz, personal observation). Transfer of antiaphrodisiac pheromones that reduce female attractiveness has been reported for example for beetles and butterflies (Andersson et al. 2003; Schulz et al. 2008; Schlechter-Helas et al. 2011). There were no significant differences in courtship probabilities and latencies of second *O. retusus* males between plug size and age treatments. Thus, we consider it unlikely that plugs contain antiaphrodisiac.

When considering the evolution of mating plugs, the particular morphology of the genitalia system plays a largely neglected but important role. In species with one female genital opening, a mating plug needs to decay before oviposition, whereas in animal groups with separate copulatory and oviposition ducts, such as most Lepidoptera and Araneae, long-lasting plugs applied to the copulatory ducts may easily evolve. However, spiders are special since most species exhibit two copulatory ducts in the female genital tract (Uhl et al. 2010). Females may allow one or two insertions by a particular male thereby allowing him to plug one or two ducts. This important morphological component and the resulting possibilities for female mate choice require being included into models on the evolution of mating plugs (e.g. Fromhage 2011).

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Conflict of interest The authors declare that there is no conflict of interest.

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Paired and complex copulatory organs: do they really impede flexible use?

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Paired and complex copulatory organs: do they really impede flexible use?

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Short title: Flexible use of paired and complex copulatory organs

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Abstract

Animals with internal fertilization possess complex male genitalia that are characterized by rapid and divergent evolution. Such complexity and divergence renders male genitalia highly species specific. Furthermore, in species with paired genitalia in males and females as is typically the case in spiders, the genital complexity is considered to restrict usage of each male intromittent organ (pedipalp) to a specific genital opening of the female genitalia. Here, we report cases of flexible pedipalp insertion (16%) in the dwarf spider *Oedothorax retusus* with highly complex pedipalps but relatively simple female genitalia. A flexible insertion mode allows males of this species to react to the mating history of the female by circumventing the highly effective mating plugs and mating into the unused side. Our findings corroborate sexual selection as the underlying selective regime for the evolution of highly complex and diverse genitalia and disagree with the lock and key hypothesis.

Keywords: functional morphology, male mating tactic, sperm competition, sexual selection, genitalia, mating plug

INTRODUCTION

Animals with internal fertilization are generally characterized by complex male genitalia whose complexity is even more obvious when they are expanded, as is generally the case during the mating sequence (Eberhard, 1985). Consequently, genitalia are used as taxonomic characters in many species. Complex genitalia have been considered to result in a lock and key situation during mating that serves to avoid interspecific matings (Mayr, 1967: p. 89). Since male and female genitalia do not generally exhibit a close match, the rapid and divergent evolution of complex genitalia is more convincingly explained by sexual selection (Eberhard, 1996) and possibly antagonistic coevolution (Arnqvist & Rowe, 2005).

The complexity of the male genitalia has further consequences on the intraspecific level particularly in species with paired sperm transfer organs where the usage of two structures that work either jointly or independently has to be coordinated during the mating sequence. Paired copulatory organs occur in spiders (Araneae: Foelix, 2011), Ephemeroptera (Gaino & Rebora, 1995; Molineri, 2010; Scudder, 1971), crustaceans (Bauer, 1986; Gruner, 1993: pp. 503, 546), Diplopods (Meisenheimer, 1921: p. 137; Wojcieszek et al., 2011), squamate reptiles (snakes, lizards: Arnold, 1986; Austin, 1984; King et al., 2009), and fish (Elasmobranchs: Fiedler, 1991: p. 222; Pratt & Carrier, 2001). Simultaneous use of male sperm transfer organs has been reported in Diplopods, in which the male inserts his gonopods into the corresponding paired female genitalia (Wojcieszek et al., 2011; Wojcieszek & Simmons, 2011). In lizards and snakes (Arnold, 1986; Bosch, 1994; King et al., 2009), as well as in Elasmobranchs (Pratt & Carrier, 2001) males generally insert their paired organs in succession into an unpaired female genital opening. The successive use of such hemipenes has been considered to increase sperm transfer (Bosch, 1994). However, simultaneous intromissions of paired male genitalia have also been described in some lizards (Bosch, 1994), as well as in some elasmobranchs (Pratt & Carrier, 2001). Moreover, when females also possess paired genitalia, side specific use of the male genitalia seems a necessary consequence of the complexity of the genital structures involved. Male mayflies (Ephemeroptera) possess paired penes, however paired female genitalia (gonopores) seem to be the ancestral condition that is only present in some species (Gaino & Rebora, 1995). Millipeds (Diplopods) often possess complex paired male and female genitalia, with a high morphological accordance, as diverse projections of the male genitalia fit into specific, corresponding parts of the female genitalia (e.g. *Antichiropus variabilis*: Wojcieszek et al., 2011). Side-specific use of male genitalia seem to be particularly characteristic for spiders in which males possess paired body appendages (pedipalps) as intromittent organs that have to couple to and interact with species-specific (however, not necessarily matched) paired female outer and inner genital structures (see material and methods for details). When pedipalps interact with the female external genitalia before sperm transfer, they are inflated by hemolymph pressure, and the various

sclerites change their relative position whereby genital coupling is achieved (e.g. Grasshoff, 1973; Uhl et al., 2007). Due to the complexity of the pedipalps and the interaction with the female genitalia it has been taken for granted that the usage of each pedipalp is restricted to a particular side, i.e. to one of both copulatory openings of the female (Fromhage & Schneider, 2005; von Helversen, 1976; Huber, 1993; Nessler, Uhl & Schneider, 2007; Welke & Schneider, 2009). To date, versatile pedipalp use has been reported only from very few species with generally simple pedipalps that are accommodated in relatively simple female genitalia (e.g. Costa, Pérez-Miles & Cort, 2000; Eberhard & Huber, 1998; Fromhage & Schneider, 2005).

If the general rule of side specificity of complex pedipalps holds, it prevents flexible use of genitalia. Flexible use may be advantageous for males with a malformed or sperm depleted pedipalp. Flexible use may increase reproductive success particularly in species in which males produce mating plugs that are effectively blocking the used copulatory duct, as occurs in many spider species (Uhl, Nessler & Schneider, 2010). If a male encounters a mated female with one blocked and one unused opening, he would profit from being able to use his pedipalps flexibly according to the state and degree of filling of his pedipalps and the female's mating history).

In the course of a study on the parameters that determine the efficacy of mating plugs in the entelegyne dwarf spider *Oedothorax retusus* (Kunz, Witthuhn & Uhl, 2014) we collected data on versatility of pedipalp use. As a strategy to reduce sperm competition *O. retusus* males produce amorphous mating plugs that block the copulatory duct (Fig. 1F-H) after usage (Kunz et al., 2014; Uhl & Busch, 2009; Uhl et al., 2014). Large and hardened mating plugs are highly effective in preventing subsequent mating into the blocked copulatory duct (Kunz et al., 2014). The standard pedipalp insertion mode in the *O. retusus* (Fig. 1A, supplementary video) is successive and ipsilateral (see material and method). We staged mating trials with two successive males - each of which had the same pedipalp amputated before the trial. We used the presence of a plug on the opposite copulatory duct after double mating as evidence of a contralateral mating and thus for versatile pedipalp use.

MATERIAL

Spider genitalia

In spiders, the secondary male genitalia are modified tips of the pedipalps that are situated between chelicerae and walking legs and are primarily used for inspecting the ground and prey. In females, pedipalps kept this function, but in males sperm are transferred from the testis into the spermophore, a storage compartment inside the terminal part of the pedipalp, the genital bulb (Foelix, 2011). From here, sperm are transferred to the female during copulation and are kept inside the female spermatheca for later use. Spider genital bulbs are relatively simple in most haplogyne

spiders but highly complex in most entelegyne spiders (Eberhard, 2010; Eberhard & Huber, 2010). In both groups, however, even closely related species can be distinguished by means of male pedipalp and female genital characters. Spider species identification is strongly based on male pedipalp morphology since its traits are easily accessible. In many haplogyne spiders the pedipalps are inserted simultaneously into a single female copulatory opening during mating (e.g. Uhl et al., 1995), whereas in the entelegyne spiders, the pedipalps are mostly used in succession, e.g. the first insertion with the right pedipalp and the second insertion with the left pedipalp, each into an assigned copulatory opening of the female. In most entelegyne spiders, the insertion mode is ipsilateral with the right pedipalp being inserted into the right copulatory duct and the left pedipalp into the left duct (von Helversen, 1976). However, due to different mating positions (parallel or antiparallel) males of some species insert crosswise, but still in the ipsilateral mode (von Helversen, 1976; Huber & Senglet, 1997). Particularly in entelegyne spiders, the female counterparts exhibit species-specific external coupling structures and likewise paired genitalia generally with two separate copulatory ducts leading to separate spermathecae of varying numbers and shapes (Herberstein et al., 2011; Uhl, 2002).

Study species *Oedothorax retusus* (Westring, 1851)

In *O. retusus* males approach the female in her web during courtship. When in antiparallel mating position, the male successively pushes his pedipalps towards the female genital openings in the ipsilateral mode (Fig. 1A, supplementary video). As soon as he succeeds in coupling to the female genital opening, his pedipalp is rotated (see video), and the membranous part of his pedipalp (hematodocha, Fig. 1A,E) inflates, and sperm transfer starts (Kunz, Garbe & Uhl, 2012). In a study on the efficacy of mating plugs in which females were mated with two males in succession (Kunz et al., 2014) we were able to explore if the insertion mode is static or flexible. In order to force the second male to use the genital opening that was plugged by the previous male, we amputated one pedipalp of each male (both right or both left). We analysed females under a dissecting microscope after their first mating, to check for the presence of a mating plug. After their second matings, female opisthosomata were fixed in Dubosq-Brazil solution (Mulisch & Welsch, 2010) to investigate the presence, position, and state of the mating plugs under the SEM (Zeiss: EVO® LS 10) (Kunz et al., 2014). The presence of plugs on both copulatory ducts after double mating is considered evidence of a contralateral mating and thus for versatile pedipalp use of second males.

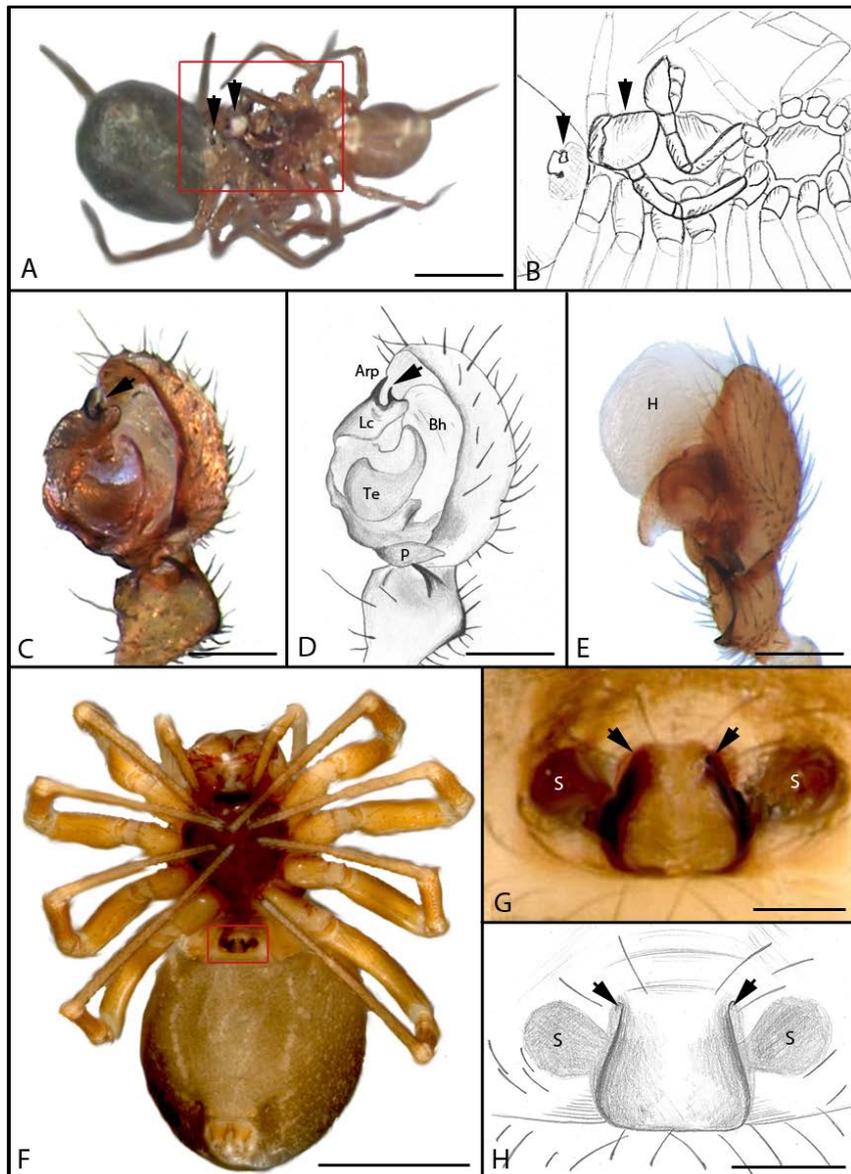


Figure 1. — (A) Mating position of the dwarf spider *Oedothorax retusus* as viewed from above: The male's right pedipalp (right arrowhead) just detached from the female's right genital opening (ipsilateral), after producing a mating plug (left arrowhead). Red square: (B) Schematic drawing of the right male's pedipalp with extended hematodocha (right arrowhead), and the corresponding mating plug (left arrowhead) in the right female genital opening. (C) Multifocus photograph (Zeiss: SteREO Discovery V20) of the left pedipalp of an *O. retusus* male. (D) Schematic drawing of an *O. retusus* male pedipalp. C-D The arrowhead marks the embolus. (E) Multifocus photograph (Zeiss: SteREO Discovery V20) of the right pedipalp of an *O. retusus* male with slightly expanded hematodocha (H). (F) Female *O. retusus* as viewed from ventral: the red square marks the female genital region (epigynum). (G) Epigynum of an *O. retusus* female. (H) Schematic drawing of the epigynum of an *O. retusus* female. G-H Arrowheads mark the genital openings. Scale bars: A,F: 1mm, C-E,G-H: 100 μ m. Arp: Anterior radical process, Bh: Basal hematodocha, C: Cymbium, Lc: Lamella characteristica, P: Paracymbium, S: Spermatheca, Te: Tegulum.

RESULTS

In our double mating trials (N= 191) with males that possessed only one pedipalp, 67.5 % of plugs produced by the first male prevented females from remating (Kunz et al., 2014). Of those that successfully coupled to the female genitalia (N= 62), 10 males (16.1%) used the contralateral, unplugged opening resulting in mating plugs on both sides (Fig. 2). The results are not due to amputation error since re-inspection of the males confirmed that the two successive males per female were lacking the pedipalps on the same side. Interestingly, males that inserted contralaterally performed more copulation attempts, and thus had longer copulation latencies than those males that were able to insert into the plugged side in the standard ipsilateral mode, however, non-significantly so (Table 1). The contralateral insertions into unused genital openings resulted in significantly longer copulation durations than ipsilateral couplings to plugged genital openings (Table 1). The number of hematodochal expansions (generally indicative of sperm transfer) was significantly higher for males that avoided the plugged female genital opening (Table 1). Further, the resulting mating plugs on the contralateral side did not differ in size or appearance from those that resulted from ipsilateral pedipalp use by the first male (Fig. 2). During contralateral matings, 80% of the females started moving around, and dragged the coupled male along (Table 1). This suggests that the contralateral insertion mode resulted in a stable coupling mechanism between pedipalp and epigyne. During conventional, ipsilateral insertions 39% of females became restless (Table 1), significantly fewer than during contralateral insertions.

Table 1. — Copulation parameters of *Oedothorax retusus* males that mated with plugged females. Data are given for the cases in which males used the plugged female genital opening (regular ipsilateral insertion) or the virgin genital opening (unexpected contralateral insertion). Data are given as events (proportions), mean \pm standard deviation, and median [interquartile range]. Statistical analysis was performed using Chi²-test, Mann-Whitney-U-tests (#), and T-tests (*).

		plugged genital opening (ipsilateral insertion)		virgin genital opening (contralateral insertion)		Z [#] , T [*] , Chi ²	P
		N	Events Mean/Median	N	Events Mean/Median		
copulation attempts	(yes:no)	52	47:5 (90.4%)	10	9:1 (90.0%)	0.001	0.970
copulation attempts	[#]	47	12 [28]	9	24 [65]	-1.38 [#]	0.169
copulation latency	[s]	52	767.4 \pm 605.8	10	928.7 \pm 876.4	-0.72 [*]	0.477
copulation duration	[s]	52	135.0 \pm 100.9	10	209.8 \pm 49.5	-3.57 [*]	0.001
hematodocha-expansions	[#]	51	81.1 \pm 62.5	10	128.9 \pm 28.9	-3.78 [*]	0.001
female behaviour	(restless:calm)	52	20:32 (38.5%)	10	8:2 (80.0%)	5.84	0.016

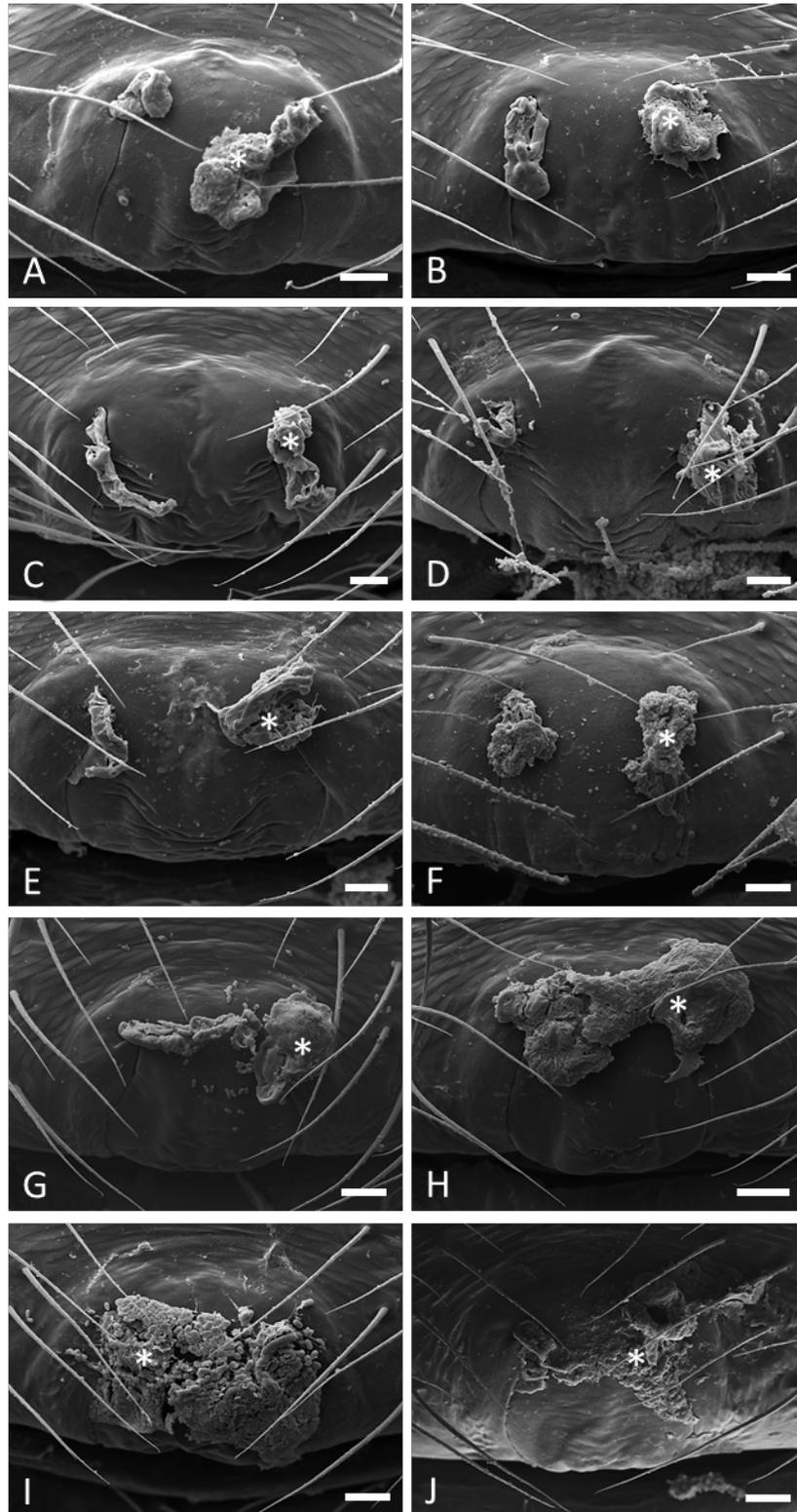


Figure 2. — A-J. SEM pictures of the genital region of *Oedothorax retusus* females with two males that both had one pedipalp amputated on the same side. Asterisks mark the mating plug that must have been produced by the second male during unexpected contralateral insertions. Scale bars: 20 μm .

DISCUSSION

Our data demonstrate that flexible use of copulatory organs is possible despite their complexity. For spiders, it was taken for granted that the generally high complexity of genitalia in entelegyne spiders limits the usage of each pedipalp to a specific female genital opening (e.g. Fromhage & Schneider, 2005; von Helversen, 1976; Nessler et al., 2007; Welke & Schneider, 2009). For spiders with simple genitalia such as theraphosids insertions via the single genital opening into any of the female receptacles were reported previously (Costa et al., 2000). The only previously known case of flexible pedipalp use in an entelegyne spider with complex pedipalps was documented for the theridiid spider *Tidarren cuneolatum* (Knoflach & van Harten, 2000). In this species, males amputate one pedipalp after their final moult and when confronted with a female that had previously mated once, they avoid the used spermatheca and insert into the virgin side for their single insertion. The simple female genital structures seem to allow such flexible use (Knoflach & van Harten, 2000). The relative mating success of males in terms of copulation duration, sperm and relative paternity awaits investigation. Similarly, the genitalia in *O. retusus* are complex in males but relatively simple in females (Fig. 1). The proportion of *O. retusus* males that shifted the insertion mode was 16%, which may suggest that the shift comes at a cost. Those *O. retusus* males that shifted the insertion mode performed slightly more copulation attempts and had longer copulation latencies before they were able to couple compared with males that used the ipsilateral side that was plugged due to a previous mating. However, once coupled, contralateral matings were more successful than ipsilateral matings into plugged openings since the former males had significantly longer copulation durations and more hematodocha-expansions (Table 1). This strongly suggests that males that used their pedipalps flexibly transferred more sperm by mating into the unused side. That the contralateral insertions result in sperm transfer is also corroborated by the fact that these males produced plugs. Plug material is placed onto the genital opening only after sperm transfer (Uhl et al., 2014). Moreover, contralateral insertions resulted in stable connections between the pedipalp and the female genitalia, since males were dragged along with their pedipalp still inserted when females became restless. Female restlessness and dragging of males also occurs during ipsilateral insertions (Kunz et al., 2012; Maes et al., 2004), however, not as frequent as during contralateral insertions. Possibly females react against the unusual insertion pattern and/or test the coupling efficacy of unconventionally inserting males.

A flexible insertion mode in animals with bilateral male and female genitalia seems to entail a high selective advantage, since it allows the male to react to their own condition and to female mating history. The former is apparent when e.g. a pedipalp is damaged due to moulting problems or predatory attack. The latter is particularly advantageous in plug producing species. By using the unmated genital opening male *O. retusus* avoid mating into a plugged genital opening that would

result in little or no paternity success in the majority of cases (Kunz et al., 2014). In contrast, males can expect about 50% paternity when avoiding the plugged opening and mating into the virgin opening instead (Kunz et al., 2012). Further, a flexible insertion mode helps to avoid direct competition between own and rival sperm inside the same spermatheca as seems to be the case in the one-palp spider *T. cuneolatum* (Knoflach & van Harten, 2000). In comparison, flexible insertion modes in snakes and lizards may not help to circumvent mating plugs but increase the probability of removing the plug from the single female genital opening. It was suggested that a male might be able to remove the plug with one hemipenis and perform copulation with the other (Bosch, 1994).

In conclusion, our study demonstrates a flexible insertion mode in a species with assumed side-specificity with pronounced consequences for male reproductive success. We postulate that flexible insertion modes are particularly advantageous for circumventing mating plugs, as is the case in our study species. Consequently, we require investigations on the frequency of fixed versus versatile use of paired copulatory organs across animal groups and on the effects of flexible use on relative fitness of the males. As for spiders, we suspect that more cases of flexible pedipalp use will be observed once the notion of a fixed insertion pattern has been abandoned. Clearly, the case of the dwarf spider *O. retusus* as demonstrated here does not support the lock and key situation since female genitalia are much simpler than male genitalia, which possibly allowed successful unconventional insertions to evolve in the first place. Strictly speaking, the key also matches the mirror image of the lock in the investigated spider species.

Overall, our study corroborates that sexual selection has been the underlying selective regime for the evolution of highly complex and diverse genitalia in species with internal fertilization (Eberhard, 1985, 1996) and that genitalic recognition via a lock and key mechanism cannot convincingly explain the evolution of genitalic complexity in spiders as well as in other animal groups.

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SUPPLEMENTARY MATERIAL

Video — Conventional mating with ipsilateral insertion mode in *Oedothorax retusus*. The male's right pedipalp just detached from the female's right genital opening (ipsilateral), after producing a mating plug. The male then inserts his left pedipalp into the left genital opening (ipsilateral).

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2.6 Contribution to manuscripts

Kunz, K., Michalik, P. & Uhl, G. (2013). **Cephalic secretion release in the male dwarf spider *Oedothorax retusus* (Linyphiidae: Erigoninae): An ultrastructural analysis.** Published in *Arthropod Structure & Development* 42, 477-482.

Data acquisition: 100%, statistical analyses: 75%, Figures/tables: 66%, Text: 75%

Kunz, K. & Uhl, G. (submitted). **Short-term nutritional limitation affects mating behaviour and reproductive output in dwarf spiders.** Submitted to *Ethology*.

Data acquisition: 100%, statistical analyses: 100%, Figures/tables: 100%, Text: 75%

Uhl, G., Kunz, K., Vöcking, O. & Lipke, E. (2014). **A spider mating plug: origin and constraints of production.** Published in *Biological Journal of the Linnean Society* 113, 345–354.

Data acquisition: 100% for SEM, statistical analyses: 100%, Figures/tables: 43%, Text: 25%

Kunz, K., Witthuhn, M. & Uhl, G. (2014). **Do the size and age of mating plugs alter their efficacy in protecting paternity?** Published in *Behavioral Ecology and Sociobiology* 68, 1321-1328.

Data acquisition: 40%, statistical analyses: 75%, Figures/tables: 66%, Text: 75%

Kunz, K., Witthuhn, M. & Uhl, G. (in revision). **Paired and complex copulatory organs: do they really impede flexible use?** In revision: *Journal of Zoology*.

Data acquisition: 40%, statistical analyses: 100%, Figures/tables: 100%, Text: 75%

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