

Causes and consequences of external female genital mutilation

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Pierick Mouginot

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Dekan: Prof. Dr. Werner Weitschies

1. Gutachter: Prof. Dr. Gabriele Uhl

2. Gutachter: Prof. Dr. Klaus Reinhardt

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Abstract

Sexual selection favours traits that confer a competitive advantage in access to mates and to their gametes. This results in males evolving a wide array of adaptations that may be conflictual with female's interests and even to collateral negative effects on female's lifespan or reproductive success. Harmful male adaptations are diverse and can be extreme. For example, males of various species evolved adaptations that incur physical damage to the female during copulation, referred to as traumatic mating. Most of these adaptations provide males with a competitive fertilization advantage due to the injection of sperm or non-sperm compounds through the wound. In the spider taxonomical literature, alterations of external genital structures have been reported in females and may result from male inflicted damage during copulation. Contrarily to other cases of traumatic mating, the transfer of sperm or non-sperm compounds does not seem to be the target of selection for external female genital mutilation (EFGM) to evolve. Therefore, investigating EFGM may provide valuable information to extend our understanding of the evolution of harmful male adaptations. In this thesis, I explore this newly discovered phenomenon and combine empirical and theoretical approaches to investigate the causes and consequences of EFGM evolution from male and female perspectives. My findings suggest that EFGM is a natural phenomenon and is potentially widespread throughout spider taxa. I demonstrate the proximal mechanism by which the male copulatory organ mutilates the external female genitalia during genital coupling and show that the mutilation results in full monopolization of the female as mutilated females are unable to remate. Using a theoretical approach, I investigated the conditions for the evolution of EFGM. The model developed suggests that EFGM evolution is favoured for last male sperm precedence and for costs to females that can be relatively high as the male-male competition increases. I present the results of physiological measurements that suggest there is no physiological cost of genital mutilation resulting from healing and immune responses for the female. Finally, I report the results of a behavioural experiment that suggest that females have control over the mutilation and selectively allow or avoid mutilation. These findings suggest that EFGM benefits males by securing paternity, that males and females may have evolved to reduce the costs incurred by the female and that female choice may also play a role in EFGM evolution.

1. Introduction

1.1. Background

Sexual selection arises from competition over mates and their gametes and favours traits that confer a competitive advantage in access to mates and fertilizations (Andersson 1994). The competition for fertilization occurs whenever there is sexual reproduction, regardless of the form of sexual reproduction (Matsuda and Abrams 1999). The competition occurs within a sex, and in anisogamous organisms with two separate sexes, sexual selection is commonly stronger in males (Bateman 1948, Andersson and Iwasa 1996). However, recent interest in sexual competition in females highlighted the occurrence of sexual selection in females (Clutton-Brock 2007, 2009, Rosvall 2011, Kvarnemo and Simmons 2013). There are two main types of traits favoured through different mechanisms of sexual selection (Andersson and Iwasa 1996). Traits can confer an advantage in gaining access to mates in direct contests or scramble competition independently of the mates. Alternatively, these traits can confer a competitive advantage that is mediated by the other sex by increasing attractiveness to the mates.

Obtaining mates represents only one part of the picture. A mating does not necessarily lead to fertilization and the competition continues after copulation for the fertilization of a set of ova (Parker 1970, 1998). As a consequence, mechanisms of sexual selection before mating, i.e. male-male competition and female choice, have their equivalents after sperm transfer in the form of sperm competition and cryptic female choice. Post-copulatory sexual selection can favour traits that result in a competitive fertilization advantage in competition between sperm of rival males (Parker 1970). Alternatively, it can favour traits that provide an advantage through differential fertilization success between sperm resulting from female morphology, physiology or behaviour (Eberhard 1996).

Obviously, the prerequisite for post-copulatory sexual selection to occur is that females mate with multiple males. Copulations with two or more males may occur simultaneously, as in organisms with external fertilization, or sequentially, as in organisms with internal fertilization. Historically, females were thought to increase their reproductive success by choosing males of good quality rather than by acquiring additional mates (Bateman 1948). However, polyandry is ubiquitous

across taxa and more common than previously thought (Kvarnemo and Simmons 2013). Polyandry can increase female reproductive success because of direct and indirect benefits (reviewed in Møller 1998, Arnqvist and Nilsson 2000, Jennions and Petrie 2000). Direct benefits such as courtship food, ejaculate nutrients or higher probability of fertilization affect female reproductive success by increasing life span or fertility. Indirect benefits are genetic benefits that affect a female's fitness via her offspring by increasing the genetic quality or diversity of the offspring. However, although beneficial for females, polyandry results in spatial or temporal overlap of ejaculates from multiple males. The competition between ejaculates reduces the paternity share for a given male and thus leads to selection for adaptations to sperm competition. Sperm competition influences a broad variety of different sexual traits in males and females (Wigby and Chapman 2004). In males, sperm competition favours adaptations that increase competitive fertilization success and thus paternity share. These male adaptations range from adaptations for engagement in sperm competition when ejaculates directly compete for fertilizations to adaptations for avoidance of sperm competition (Simmons 2001). Adaptations for engagement in sperm competition enhance the competitive ability of ejaculates, whereas adaptations for avoidance of sperm competition allow males to monopolize females and their ova. Among adaptations to avoid sperm competition, some benefit males by altering female physiology, behaviour or anatomy. For example, males evolved a wide array of adaptations that manipulate the frequency of female remating by behavioural, biochemical or physical mate guarding (Simmons 2001, 2014). Males can guard females before and after mating to keep rival males at bay. However, this imposes a trade-off due to costs in terms of male's inability to mate with additional females when guarding. Males can avoid these costs by guarding females without being physically present. Males can transfer seminal fluid proteins that reduce female receptivity (Wigby and Chapman 2005, Fricke et al. 2009) or anti-aphrodisiac pheromones that reduce female attractiveness to other males (Gilbert 1976). Males can also apply physical barriers to the female reproductive tract in the form of a mating plug that impedes access to female genitalia. Mating plugs can consist of secretions transferred together with the ejaculate that hardens and cements female genitalia, or even a part or the entire male copulatory organ that breaks-off and remains

in the female genital tract (Uhl et al. 2010). These male adaptations to sperm competition may affect female fitness when multiple mating is beneficial for females (Parker 1979, Stockley 1997). When the evolutionary interests of males and females do not converge, male and female optima cannot be realized simultaneously (Parker 1979). This implies that adaptations in one sex may shift the other sex from its optimum, and impose a “lag load” (Rice et al. 2006) (also termed “conflict load” Lessels 2006). The lag load represents a fitness reduction in the other sex which generates an evolutionary response to reduce the fitness cost. Sexual conflict theory predicts antagonistic coevolution between male adaptations and female counter-adaptations when the male trait is advantageous to males but disadvantageous to females (Parker 1979, 2006). Alternatively, females may gain indirect benefits through sons that inherit the father’s trait. If the indirect benefits outweigh the direct costs inflicted by the male trait, females may “gain by losing” (Eberhard 2005). In this view, female resistance to a male trait may represent a female choice for antagonistic male adaptation (Cordero and Eberhard 2003). Sexual conflict is recognised as an important evolutionary engine of male and female traits. It occurs across virtually every taxa and over every facet of reproduction and has strong potential to explain the evolution of manipulative adaptations that seem deleterious to the other sex (Parker 1979, Arnqvist and Rowe 2005, Kokko and Jennions 2014). Manipulative adaptations may entail additional deleterious effects above the lag load referred to as “harm”. These negative effects on female fitness sum up with the lag load to constitute the total cost of the male adaptation incurred to females (Johnstone and Keller 2000, Morrow et al. 2003, Lessels 2005, 2006). However, these costs can have opposite effects on the fitness of the manipulative males. Indeed, as the manipulation reduces the male’s lag load, it may also affect male fitness negatively through the negative effect of harm on female’s reproductive output. This makes the evolution of harmful male traits counterintuitive. In the literature, harmful traits are explained either as being specifically adapted to cause harm, or as a side effect of an adaptation to male-male competition (Morrow et al. 2003). The first hypothesis, termed the “adaptive harm” hypothesis, states that a male benefit from a trait specifically adapted to harm females. In this sense, the harm would be the target of selection. The question whether males can gain benefits through inflicting harm has been addressed with theoretical models that showed that males may benefit if females respond to the harm by decreasing their remating rate or by

increasing their oviposition rate (Lessells 1999, Johnstone and Keller 2000, Lessells 2005). However, the assumption that a male harmful trait may be advantageous due to the effect of harm on the female's physiology or behaviour remains theoretical. Indeed, empirical studies revealed no evidence that harm per se was causally responsible for increasing male fitness (Morrow et al. 2003, Hotzy and Arnqvist 2009, Grieshop and Polak 2014). The adaptive harm hypothesis is therefore not supported for the evolution of this male harmful trait. Alternatively, the "pleiotropic harm hypothesis", states that the harm is a negative side effect of an adaptation which gives a reproductive advantage in male-male competition (Parker 1979). In other words, the male gains benefits from a trait despite the associated detrimental effect on the female. The harm is not the target of selection and the trait evolved for reasons other than causing injury per se. In this case, selection on both sexes to reduce the harm imposed upon females is expected (Morrow and Arnqvist 2003). Such additional cost, also named "collateral harm", is the most supported hypothesis for explaining harmful traits.

Research on the evolution of harmful male traits has been fuelled by two renowned cases. First, toxic seminal fluid proteins that are transferred together with the ejaculate (Chapman et al. 1995, Gems and Riddle 1996). These seminal fluid proteins reduce the remating rate and increase the egg-laying rate of females but also negatively affect their lifespan (Chapman et al. 1995, Fricke et al. 2009). A second case is internal genital damage incurred by the male spiny copulatory organ to the female genital tract (Crudginton and Siva-Jothy 2000, Blackenhorn et al. 2002). When the physical damage is incurred by the partner via specialized devices during copulation, it is referred to as "traumatic mating" (Lange et al. 2013, Reinhardt 2015). Traumatic mating has been suggested to positively affect male fitness due to direct benefits of physical anchorage during copulation, fecundity stimulation, fertilization efficiency or paternity benefits (Lange et al. 2013). However, research on traumatic mating has revealed the role of fluids transfer in the selection process of traumatic mating. Indeed, male benefits are always linked to injection of sperm or non-sperm compounds via the wound, termed respectively traumatic insemination or secretion transfer (Reinhardt et al. 2015). For example, in the case of internal genital damage, the selective advantage of the damage is mediated by the effect of seminal fluids that are transferred through the wound (Hotzy et al. 2012, Yamane et al. 2015). The physical damage that a female experiences

may involve costs in terms of wound healing, infection or intrusion of non-self-particles that may require resource re-allocation into repair and immune responses (Moret and Schmid-Hempel 2000). These additional costs are considered as the collateral harm of male traumatic mating traits and are expected to be selected against in both sexes (Morrow et al. 2003). The combination of different selection regimes acting on the harm and lag load of a male trait in males and females may lead to several evolutionary outcomes. Females may respond to male harmful traits by resistance or tolerance (Svensson and Råberg 2010, Reinhardt et al. 2015). Resistance traits will reduce the costs incurred by avoiding or minimizing the male trait. This female defence strategy causes costs to males in terms of lag load and induces antagonistic coevolution. As an example, females of some bruchid beetles evolved thickened sclerotized genital tracts in response to punctures of the genital tract caused by the male's spiny penis (Rönn et al. 2007). Alternatively, tolerance traits will reduce the costs incurred by minimizing the fitness impact of the male trait. This female defensive strategy does not cause costs to males, but rather reduces the harm, and perhaps allows for evolutionary novelties (Reinhardt et al. 2015). As an example, female bed bugs evolved extra genital structures that receive the male genital organ in response to traumatic insemination by the male genital organ into the body cavity (Morrow and Arnqvist 2003). As a consequence, sexual conflict over harmful male adaptation may lead to trait exaggeration, simplification or disappearance and thus, result in increased genital diversity in both sexes across species. Reinhardt (2016) noted that examples of traumatic mating whose benefits are not mediated by fluid transfer are scarce and that such cases may challenge the current understanding of traumatic mating and therefore, represent a good system to study the evolutionary forces driving harmful male adaptations.

Arthropods are particularly prone to post-copulatory sexual selection and sexual conflict since they combine female multiple mating and female sperm storage organs, which are diverse both in number and characteristics (Simmons 2001). In spiders, selection via sperm competition and cryptic female choice has led to the evolution of diverse and striking adaptations in males and females. Female genital morphology has been suggested to be crucial for the evolution of these adaptations in the way that it sets the rules for male investment strategies (Eberhard 1996, Schneider and Andrade 2011). For example, the morphology of the female's sperm storage organ

may set the sperm precedence pattern in the sequence of mating males or allow females to control relative paternity, which will influence what kind of trait may give a selective advantage to males (Schneider and Andrade 2011). As another example, in most spider species, copulatory and oviposition ducts are separated in females (Foelix 2011), thus, allowing males to plug copulatory ducts without affecting oviposition. Therefore, the internal female genital morphology may have provided an evolutionary route resulting in the high prevalence and diversity of mating plugs in spiders (Uhl et al. 2010). In entelegyne spiders, male copulatory organs are highly differentiated and show a correlation of shapes with the diverse and often complex external genitalia of females (Eberhard 2004, Foelix 2011). Copulatory organs of spider males, the pedipalps, are paired and possess complex sclerotized structures that function to contact and brace against the female's external genital structures during copulation. The female copulatory organ is a sclerotized plate with several cuticular extensions (Foelix 2011). During copulation, these external genital structures function as an anchoring device for the male copulatory organs and are essential to the copulatory mechanism (Grasshoff 1973). The evolution of the copulatory structures on the female external genitalia may have promoted the evolution a harmful male adaptation. Indeed, structures of the external female genitalia have been reported lacking for several species and genera in the taxonomic literature (Figure 1). The alteration of external female genitalia may be the result of an unexplored and potentially widespread male strategy. Males may damage the external female genitalia by removing the female structures necessary for genital coupling. As a consequence, external female genital mutilation (EFGM) may hinder females from remating with subsequent males. From the male perspective, EFGM is advantageous since it allows female monopolization. Moreover, EFGM does not entail the costs of missing mating opportunities that males suffer from in the case of investment in behavioural guarding (Hasselquist and Bensch 1991) or in a copulatory plug consisting of their own copulatory organ or entire body (Parker 1984, Fromhage 2012). From the female perspective, EFGM may hinder polyandry and result in a lag load. Additionally, the damage caused by the genital mutilation may entail harm to the female in terms of haemolymph loss or infection risk. In my doctoral thesis, I explore EFGM and investigate the causes and consequences of its evolution in the orb-web spider *Larinia jeskovi* (Marusik 1986).

Species	External female genitalia		Species	External female genitalia	
	Intact	Altered		Intact	Altered
<i>Mangora itza</i> ¹			<i>Cyclosa omonaga</i> ⁶		
<i>Aculepeira packardi</i> ²			<i>Cyclosa confusa</i> ⁶		
<i>Larinia chloris</i> ³			<i>Cyclosa japonica</i> ⁶		
<i>Larinia tabida</i> ⁴			<i>Cyclosa atrata</i> ⁶		
<i>Larinia jeskovi</i> ^{5,6}			<i>Cyclosa hamulata</i> ⁶		
<i>Larinia elegans</i> ⁵			<i>Cyclosa maritima</i> ⁶		
<i>Larinia bonneti</i> ⁵			<i>Cyclosa mulmeinensis</i> ⁶		
<i>Larinia bonneti</i> ⁶			<i>Cyclosa vallata</i> ⁶		
<i>Larinia argiopiformis</i> ⁶			<i>Cyclosa argenteoalba</i> ⁶		
<i>Larinia fusiformis</i> ⁶			<i>Cyclosa okumae</i> ⁶		
<i>Larinia phthisica</i> ⁶			<i>Cyclosa ginnaga</i> ⁶		
<i>Anepsion japonicum</i> ⁶			<i>Cyclosa kumadai</i> ⁶		
<i>Elleguna major</i> ⁸					

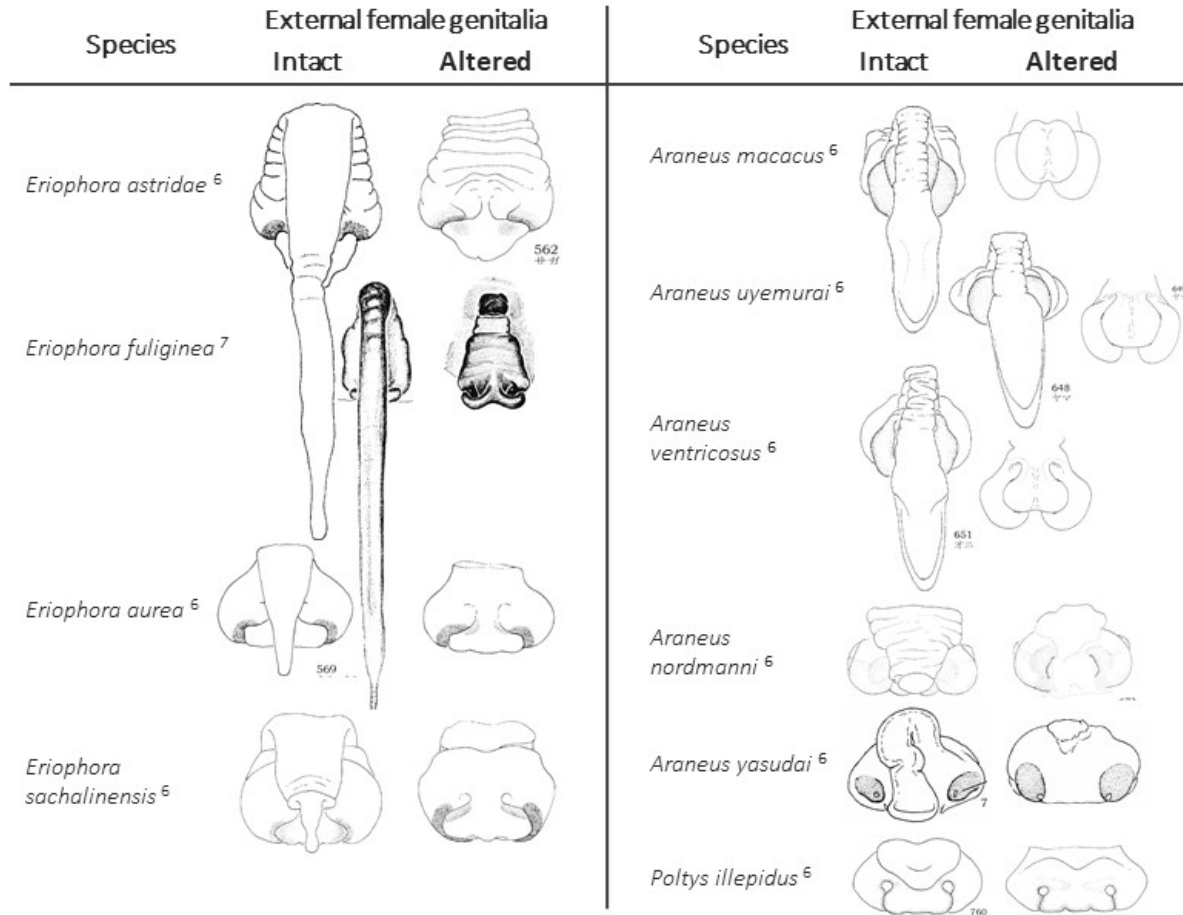


Figure 1: External female genitalia drawings in spider species of the family Araneidae for which altered structures have been reported in the taxonomic literature. The first column gives the species name and reference number, the second column shows intact female genitalia representing the diversity of external genitalic structures, the third column shows altered female genitalia representing the ubiquity of EFGM in spiders. Numbers refer to the taxonomic references: 1, Levi 2005; 2, Levi 1977; 3, Levi 1986; 4, Grasshoff 1970; 5, Szinetár and Eichardt 2004; 6, Tanikawa 2007; 7, Levi 1970; 8, Gray & Smith 2008.

Larinia jeskovi is an araneid spider distributed from Japan to central Europe (Marusik 1986, Tanikawa 1989, Kupryjanowicz 1995, 1997, Szinetár 2000, Szinetár and Eichardt 2004). The species occurs in wetland areas like swampy meadows or reed beds. The mating season extends from the end of July until the end of August. Females produce egg sacs after the mating season in September, and the juveniles are already present in October (Szinetár and Eichardt 2004). During the mating season, individuals are active at night. Females build a typical orb-web at sunset that they dismantle before dawn. Adult males are found wandering and occasionally around or on

female webs while females are found at the hub of their web (Szinetár 2000). Copulation takes place on the female web. As in typical araneid spiders, copulation is preceded by courtship. Initially, the male remains at the periphery of the web and uses its front legs to pluck threads, thus producing vibrations on the female web (Foelix 2011). Males perform several approaches towards the female and attach a thread onto the female's web from the female location to the edge of the web, named "mating thread". The male then producing vibrations on the mating thread until the female moves onto it. The receptive female and the male move toward each other and, after exchanging repeated contacts of their forelegs, may assume copulatory posture. During copulation, males insert their paired copulatory organs alternately in the paired female copulatory openings that lead to paired spermathecae. The external genitalia of *L. jeskovi* females possess an anchoring device, the scape, used by the male genital organ to achieve genital coupling. This structure may be the target of female genital mutilation in *Larinia jeskovi* (Figure 1).

1.2. Aims of the presented work

External genital mutilation in spiders has been observed in females and may be the result of a male adaptation. This phenomenon is unexplored and may represent a new opportunity to investigate the evolution of traumatic male sexual adaptations. Indeed, contrary to other cases of traumatic mating male adaptations, the transfer of sperm or non-sperm compounds does not seem to be the target of selection for EFGM to evolve. Therefore, investigating EFGM may help to extend our understanding of the evolution of harmful male adaptations. The first aim of this thesis was to explore external female genital mutilation and describe the natural and potentially widespread occurrence of the phenomenon. A second aim was to investigate the causes and consequences of EFGM evolution that we considered from male and female perspectives.

In the first chapter, I present suspected cases of EFGM across spider taxa and describe its occurrence in a natural population of the orb-weaving spider *Larinia jeskovi*. I then consider the proximal mechanisms of EFGM. First, I present the investigation of the functional morphology of the copulatory mechanism. Thus, I demonstrate how male and female genitalia are coupled during copulation and reveal how males achieve the mutilation of the external female genitalia. Second,

I present a laboratory experiment that demonstrates the adaptive value of EFGM for males as a means to monopolize females after copulation.

In the second chapter, I study how EFGM, as a male adaptation that possibly harms the female may evolve. Males may gain from mutilating their partners by securing a high share of paternity, however, the mutilation costs incurred by the female may reduce the advantage of such male adaptation and hinder its evolution. Using a theoretical approach, I present the conditions under which EFGM can evolve. More generally, the aim is to provide predictions on the occurrence of EFGM regarding to the mating system, sperm precedence pattern and level of mutilation cost to the female that can be empirically tested.

In the third chapter, I consider the consequences of EFGM for the female in terms of collateral costs (harm) incurred by the female by the genital mutilation. I present a study that investigates the potential collateral costs associated with genital mutilation resulting from healing and immune responses.

In the fourth chapter, I consider the consequences of EFGM for the female in terms of lag load suffered by the female due to the post-copulatory monopolization. If females benefit from polyandry, we expect selection on females to evolve a counter-measure to male monopolization. I present a study that demonstrates that females have control over the mutilation and selectively allow or avoid mutilation by means of sexual cannibalism.

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3. Publications

Contribution to the publications

Chapter 4.1 – Mouginit, P.; Prügel, J.; Thom, U.; Steinhoff, P. O.; Kupryjanowicz, J.; & Uhl, G. 2015: Securing paternity by mutilating female genitalia in spiders. *Current Biology*, 25, 2980-2984.

Gabriele Uhl designed the study and compiled the information on potential spider species with genital mutilation given in Table S1. Pierick Mouginit, Josepha Prügel, and Ulrike Thom conducted the field study. Pierick Mouginit conducted the laboratory study and analyzed the data. Pierick Mouginit, Gabriele Uhl and Philip O.M. Steinhoff performed the cryo-fixations and micro-CT reconstruction. Pierick Mouginit and Gabriele Uhl wrote the manuscript. Janusz Kupryjanowicz provided essential logistic support.

Chapter 4.2 – Mouginit, P.; Uhl, G.; & Fromhage, L. 2017: Evolution of external female genital mutilation: why do males harm their mates? *Royal Society open science*, 4, 171195.

Pierick Mouginit and Lutz Fromhage contributed through modelling and writing. Gabriele Uhl contributed through discussions and writing.

Chapter 4.3 – Mouginit, P.; Toshkova, N., Uhl, G.; & Beaulieu, M. (in prep). Differential oxidative costs of bodily harm and genital damage in an orb-weaving spider.

Pierick Mouginit, Gabriele Uhl and Michaël Beaulieu designed the study and contributed through discussions. PM collected animals and carried out the experimental treatments. Pierick Mouginit and Nia Toshkova carried out the physiological measurements. Pierick Mouginit and Michaël Beaulieu carried out the statistical analysis. All authors contributed through writing.

Chapter 4.4 – Mouginit, P. & Uhl, G. (in prep): Female control over mutilation of their genitalia by males.

Pierick Mouginit designed the study, collected animals, conducted the laboratory study, carried out the statistical analysis and wrote the manuscript. Gabriele Uhl contributed through discussions and writing.

Supervisor: Prof. Dr. Gabriele Uhl

Student: Pierick Mouginit

3.1. Chapter 1: Securing paternity by mutilating female genitalia in spiders

Pierick Mouginot¹, Josepha Prügel¹, Ulrike Thom¹, Philip O.M. Steinhoff¹, Janusz Kupryjanowicz²,
and Gabriele Uhl¹

¹General and Systematic Zoology, Zoological Institute and Museum, University of Greifswald,
Greifswald 17489, Germany

²Andrzej Myrcha Nature Center, University of Bialystok, 15950 Bialystok, Poland

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Current Biology

Securing Paternity by Mutilating Female Genitalia in Spiders

Highlights

- Spider males remove a coupling structure on the female external genitalia
- Mutilation of female genitalia prevents harmed females from remating
- External female genital mutilation is an adaptation for securing paternity
- Mutilation of external female genitalia is a widespread but overlooked phenomenon

Authors

Pierick Mougnot, Josepha Prügel, Ulrike Thom, Philip O.M. Steinhoff, Janusz Kupryjanowicz, Gabriele Uhl

Correspondence

gabriele.uhl@uni-greifswald.de

In Brief

There are manifold ways by which males secure paternity. Here, Mougnot et al. present an obvious but as yet unexplored means of securing paternity: males remove the structure of the female genitalia that is necessary for genital coupling. This study provides the first evidence of a direct adaptive function for the mutilation of female genitalia.



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Securing Paternity by Mutilating Female Genitalia in Spiders

Pierick Mougnot,¹ Josepha Prügel,¹ Ulrike Thom,¹ Philip O.M. Steinhoff,¹ Janusz Kupryjanowicz,² and Gabriele Uhl^{1,*}

¹General and Systematic Zoology, Zoological Institute and Museum, University of Greifswald, Greifswald 17489, Germany

²Andrzej Myrcha Nature Center, University of Białystok, 15950 Białystok, Poland

*Correspondence: gabriele.uhl@uni-greifswald.de

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SUMMARY

Competition between males and their sperm over access to females and their eggs [1–3] has resulted in manifold ways by which males try to secure paternity, ranging from physically guarding the female after mating to reducing her receptivity or her attractiveness to subsequent males by transferring manipulative substances [4, 5] or by mechanically sealing the female reproductive tract with a copulatory plug [1, 3, 6]. Copulations may also result in internal damage of the female genitalia [7–9]; however, this is not considered as a direct adaptation against sperm competition but as a collateral effect [9–14]. Here, we present a drastic and direct mechanism for securing paternity: the removal of coupling structures on female genitalia by males. In the orb-weaving spider *Larinia jeskovi* [15] males remove the scapus, a crucial coupling device on the female external genital region. Reconstruction of the coupling mechanism using micro-CT-scanned mating pairs revealed that several sclerites of the male genitalia interact to break off the scapus. Once it is removed, remating cannot occur due to mechanical coupling difficulties. In the field, male-inflicted genital damage is very prevalent since all female *L. jeskovi* were found to be mutilated at the end of the mating season. External genital mutilation is an overlooked but widely spread phenomenon since 80 additional spider species were found for which male genital manipulation can be suspected. Interlocking genitalia provide an evolutionary platform for the rapid evolution of this highly effective mechanism to secure paternity, and we suspect that other animal groups with interlocking genital structures might reveal similarly drastic male adaptations.

RESULTS AND DISCUSSION

Mutilation of Female Genitalia in the Field and Laboratory

We used the orb-weaving spider *Larinia jeskovi* Marusik, 1986 to investigate whether the damage to the female (Figure 1) is caused

by males during mating. In the field, at the beginning of the mating season, 57% (12 of 21) of females were found to be mature, and 83.3% (10 of 12) of these were already missing the scapus. At the end of the mating season, all adult females (26 females) were missing the scapus. In the laboratory, we staged 40 mating trials with virgin females, of which 72.5% mated (29 of 40). Pedipalp insertions were very short, lasting for 2.48 s on average (SD = 0.81, n = 29). A randomly chosen subset of mating pairs (n = 14) were allowed to mate undisturbed by the experimenter, resulting in a median of four successive insertions of the two pedipalps that serve as secondary sperm transferring organs in spiders (interquartile range [IQR] = 0, range = 1–5). Postmating, 93% (13 of 14) of females were found without the scapus (Figure 2). Another randomly chosen subset of 15 mating pairs was only allowed a single insertion. Single insertions resulted in significantly fewer females with a broken-off scapus (1 of 15) compared to undisturbed matings ($\chi^2 = 18.23$, degrees of freedom [df] = 1, $p < 0.001$; Figure 2). These data show that external female genital mutilation in *L. jeskovi* results from mating and occurs at a high frequency and that the probability of mutilation depends on the number of insertions of the male's copulatory organs.

Mechanism of Mutilation

We cryo-fixed mating pairs of *L. jeskovi* and reconstructed the copulatory mechanism by using X-ray micro-computed tomography (micro-CT) (Figure 3A). The structures involved in coupling were segmented to visualize the mutilation mechanism: the female epigynum, with its lateral lobes and central scapus, is interlocking with various sclerites of the paired male copulatory organs, the pedipalps (Figures 3B, 3C, and 3E). While the sperm-transferring structure of the active pedipalp, the embolus, is inserted into the copulatory duct (Figure 3B), the terminal apophysis is secured under the lateral lobes and the median apophysis is inserted medially deep into the funnel-like groove of the scapus. In addition, two further sclerites grasp the scapus from the side: the so-called conductor, which is secured in its outside position by a basal-tooth-like protrusion of the median apophysis, and the tegular apophysis, which is positioned underneath the scapus opposite of the conductor (Figures 3B, 3C, and 3E). The tegular apophysis cuts the scapus like a blade (Figure 3D). Scapus removal seems to require more than one insertion, since with a single insertion the male cuts only half of the scapus (Figure 3E). Consequently, the copulatory mechanism can explain why more than one insertion is generally necessary for removal of the scapus (Figure 2). An interactive 3D PDF can be found in Data S1.

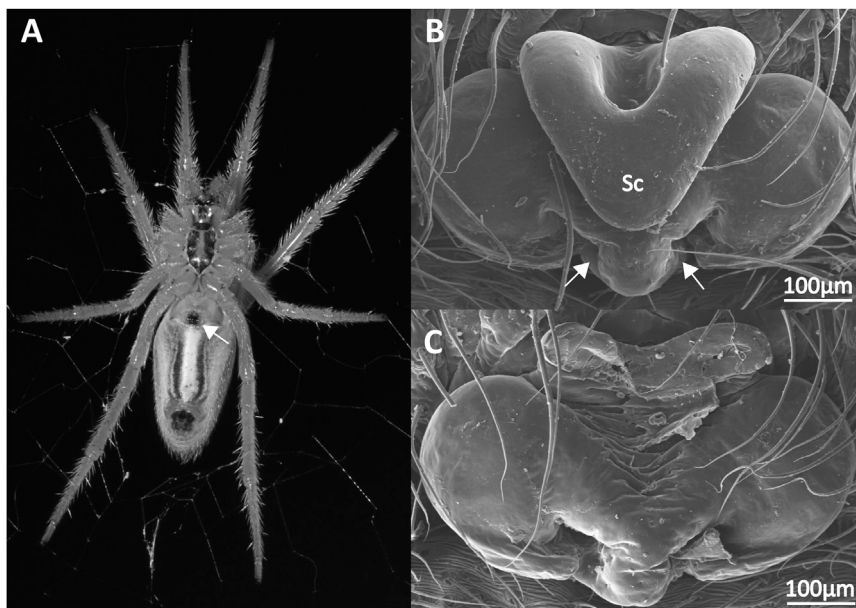


Figure 1. External Female Genital Mutilation

(A) Macro-photograph of female *Larinia jeskovi* in her web in ventral view. Arrow points to external genitalia.

(B and C) Scanning electron microscopic photographs of the external genitalia in ventral view with (B) an intact scapus (Sc) or (C) the scapus missing. Arrows point to copulatory openings. Scale bars, 100 μ m.

See also [Table S1](#) for 80 additional spider species with suspected female genital damage.

The copulatory mechanisms in orb-weaving spiders generally entail a complex grasping mechanism of the scapus by several palpal sclerites that are hooked into its grooves and are pressed against it [16, 17]. For any given species with such a tight interlocking mechanism, slight changes in male behavior, i.e., twisting behavior or slight mechanical changes through larger pressure

implied on the scapus, can result in scapus damage and removal. The resultant high fertilization success for the manipulating male would lead to selection for twisting, and the mechanism would evolve to fixation.

Remating Probability of Mutilated Females

For investigation of the effect of mutilation of the female genitalia on female remating probability, females that lost the scapus in the undisturbed mating trials ($n = 13$) and females that remained intact after the single-insertion mating trials ($n = 14$) received a second male. The proportion of pairs that performed mating attempts in second matings (22 of 27) did not differ from that of first matings (29 of 40; $\chi^2 = 0.31$, $df = 1$, $p = 0.58$). Mating attempts entail courtship by the male in the female orb web, the female approaching the male and entering a specific mating posture, and insertion attempts by the male. Remating trials with previously mutilated and non-mutilated females did not differ in the proportion of pairs that performed mating attempts (12 of 13 with a mutilated female and 10 of 14 with an intact female; Fisher's exact test, $p = 0.33$), suggesting that neither female receptivity nor female attractiveness to males was reduced by mutilation. However, none of the females with a broken-off scapus remated (0 of 12), in contrast to all females with an intact scapus (10 of 10; Fisher's exact test, $p < 0.001$; [Figure 4](#)). To control for the effect of mating experience on female receptivity, male courtship probability, and mating success, we experimentally mutilated another 14 virgin females. Mating attempts occurred in 78.6% (11 of 14) of the mating trials in the experimentally mutilated group, which is not significantly different from the overall 72.5% (29 of 40) of the two other groups with intact virgin females (Fisher's exact test, $p = 0.74$). There was only one successful mating with the experimentally mutilated females (1 of 11). Mating success of the experimentally mutilated females did not significantly differ from rematings of females mutilated during a previous mating (0 of 12; Fisher's exact test, $p = 1$; [Figure 3](#)). Our data demonstrate that willingness to mate does not decline for females postmating and that mutilated females are equally willing to remate as are intact females. Likewise, males court both virgin and mated females and do not distinguish in courtship activity between mutilated and intact females. Consequently, mating probability seems to be determined only by the presence of the scapus as the crucial interlocking structure.

Costs of Mutilation

The costs for the monopolizing male entailed in this strategy seem negligible compared to guarding the female, producing accessory seminal substances that alter female receptivity or attractiveness [18], or using body parts and secretory substances as mating plugs [6, 19, 20], all of which may severely reduce the male's future mating success [21, 22]. The transfer of mating plugs, be they broken male genitalia or secretory mating plugs, can easily lead to an arms race between males resulting in, for example, the evolution of means to remove the material when encountering an already mated female [23, 24]. However, the potential for the evolution of countermeasures in rival males is highly limited in the case of external mutilation of female genitalia. A mating mechanism that does not require the missing coupling structure, for example through traumatic insemination [25, 26], would be the only conceivable alternative.

Females may incur several types of costs from genital mutilation [2, 3]. As was shown for species that exhibit internal damage, it seems likely that external genital damage can reduce female survival and longevity or lifetime reproductive success by causing infections [27]. If *L. jeskovi* females benefit from polyandry, sexual conflict over mating rates should be strong [10, 28]. Under this scenario, females should exhibit marked mate choice and/or allow only one insertion to reduce the probability of becoming mutilated. In the laboratory, virgin female *L. jeskovi* were highly receptive and allowed an average of four insertions that lead to near 100% mutilation probability. In the field, however, female mating behavior may strongly depend on perceived availability of males that could alter female mating behavior [29]. Finally, costs for the female could be balanced by

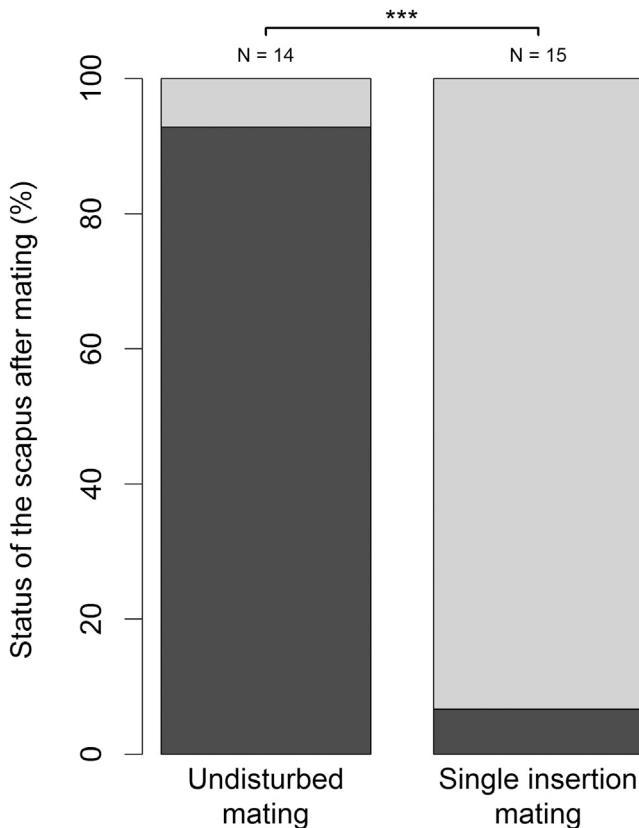


Figure 2. Mutilation Results from Copulation

Percentages of females with intact scapus (light gray) or broken off scapus (dark gray) after a mating trial that was undisturbed by the experimenter (median of four insertions) or a mating trial that was terminated by the experimenter after a single insertion.

the indirect benefit of producing sons that inherit the highly successful manipulative traits of their fathers [30].

Conclusions

Our study demonstrates that mutilation of external female genitalia is a highly effective means of securing paternity in *L. jeskovi* and represents the first evidence for the existence of male alteration of the female genital structure as a direct counter-adaptation to sperm competition. Species in which male and female structures interlock during copulation are pre-adapted for mutilation since slight changes in male behavior can result in substantial functional consequences that impact on the mating system. In fact, external female genital mutilation very likely occurs in many more spider species from diverse families, with and without scapus structures (see Table S1) and is likely to occur in other taxa with interlocking structures, for example in Odonata [31]. The costs and benefits for females and the consequential degree of sexual conflict remain to be explored.

EXPERIMENTAL PROCEDURES

Field Observations

Females of *Larinia jeskovi* were collected in August 2013 from a swamp close to Gugny in the Biebrza National Park, Poland (53°21'1.36" N, 22°34'37.45" E). Twenty-one individuals were collected at the beginning (August 15, 2013) and

26 at the end (August 27, 2013) of the mating season, and the status of the scapus was determined under a stereo microscope (Zeiss Discovery V20 Stereo Microscope, Carl Zeiss MicroImaging).

Genital Morphology

In entelegyne spiders, such as *L. jeskovi*, females possess two insemination ducts, each leading sperm to a storage site from which sperm is ultimately released via a separate fertilization duct to meet the eggs during oviposition. Eggs are then laid through the oviduct opening. The insemination ducts are situated within the so-called epigyneal plate, whereas the oviduct opening marks the posterior end of the epigynum [32]. In most entelegyne spiders, males inseminate the female by successively inserting parts of their paired sperm transfer organs, the pedipalps, into the copulatory ducts. In many entelegynes, and particularly within araneid spiders, the pedipalp consists of several sclerites and membranes. Prior to insertion, the pedipalp expands and twists, thereby moving the sclerites into specific positions. The sclerites play an essential role in coupling to structures of the epigynum [16, 17, 32–34].

Scanning Electron Microscopic Micrographs

Females with and without scapus were dehydrated in a graded ethanol series and were critical-point dried with a BAL-TEC CPD 030. The specimens were sputter coated with gold using a Polaron SC 7640 sputter coater and were investigated with a Zeiss DSM 940A scanning electron microscope.

Mutilation Mechanism: Cryofixation of Mating Pairs and Micro-CT

To explore the interlocking mechanism of male and female genitalia, we fixed several couples in copula by cryofixation. We staged a mating trial with a virgin female as described in the Mating Experiments section and fixed the couples during genital coupling by pouring liquid nitrogen (−196°C) over them. The couples were transferred to cold 80% ethanol at −40°C for several weeks to insure stable fixation [17]. The fixed couples were warmed up slowly and dehydrated in a graded ethanol series (80%, 90%, 96%, and three times in 99% ethanol for 24 hr each). The samples were then transferred to 1% iodine solution (iodine, Carl Roth) in 99.8% ethanol for two nights to enhance tissue contrast.

For micro-CT, the samples were either scanned in 90% ethanol or critical-point dried (Leica EM CPD300) and mounted on an aluminum rod with super glue. The scans were performed with an XRadia Micro XCT-200 (Carl Zeiss X-ray Microscopy). For the reconstruction depicted in Figure 3 we used a 4× and 10× object lens unit, at 40 kV and 8 W, with a pixel size of 4.65 μm and 2.25 μm, respectively. Tomography projections were reconstructed using the software provided by XRadia. For image segmentation, the software platform Amira 5.6.0 (FEI, Visualization Science Group) was used.

Mating Experiments

In the laboratory, double mating experiments were staged to assess the mutilation probability with virgin females and the remating success of mutilated females. To this aim, males and subadult females of *Larinia jeskovi* were collected in August 2014 from Biebrza National Park, Poland. Individuals were kept in individual 250 ml plastic cups, watered on a daily basis, and fed 2–3 days per week with one *Musca domestica*. The mating status of males was unknown as they were collected as adults. After their last molt, virgin females were transferred individually to plastic hexagonal boxes (18 × 18 × 6 cm) in which they built a web. We staged mating trials with 54 females. Females were randomly assigned to one of three treatment groups: (1) The female was mated with a first male in a mating trial undisturbed by the experimenter (n = 20). (2) The female was mated with a first male in a mating trial terminated by the experimenter after a single insertion (n = 20). A second male was introduced to the web of the females after 2 days to explore differential remating behavior. (3) Females were experimentally mutilated by immobilization under a net and removal of the scapus with forceps (n = 14); the mating trial was staged 2 days after the ablation of the scapus.

Mating experiments lasted for 1 hr and were started by placement of a male in an upper corner of the web. If contact between male and female began near the end of the observation period, observations were prolonged for an

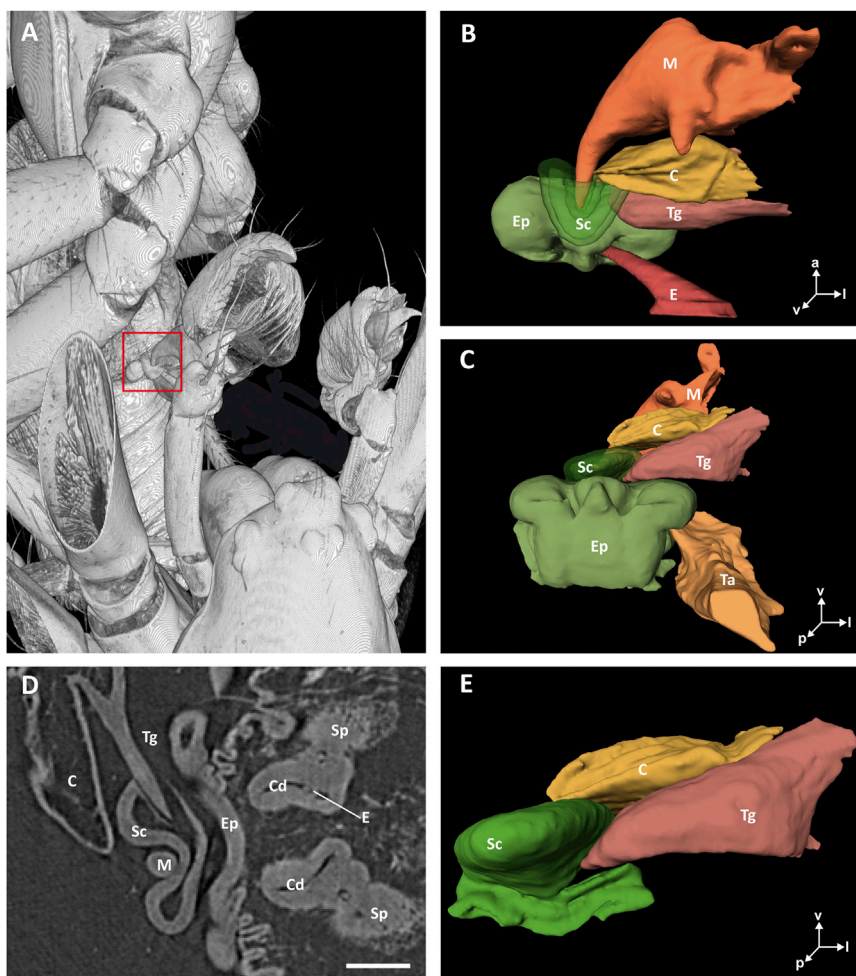


Figure 3. Mechanism of Mutilation of Female Genitalia in *Larinia jeskovi*, Obtained by High-Resolution X-Ray Tomography.

(A) Volume rendering of a *L. jeskovi* pair in copula (left, female; right, male). The male copulates with the left pedipalp.

(B) 3D reconstruction of the male and female genital structures engaged in mutilation. The median apophysis (M) of the male inserts into the groove of the female's scapus (Sc). The conductor (C) and the tegular apophysis (Tg) are securing the scapus (Sc) similar to a precision grip; the tegular apophysis (Tg) works as a chock and cuts the scapus halfway. The actual sperm-transferring structure, the embolus (E), is inserted into one of the genital openings on the epigyne (Ep).

(C) Posterior view, showing also the terminal apophysis (Ta) interlocking with the lateral protrusions of the epigyne (Ep).

(D) Virtual horizontal section (obtained by X-ray tomography) through the female scapus region of the cryo-fixed pair. The tegular apophysis slashes the base of the scapus. Cd, copulatory duct; Sp, spermatheca. Scale bar, 100 μ m.

(E) 3D reconstruction demonstrating that the base of the scapus is mutilated by the tegular apophysis.

See also [Data S1](#) for an interactive 3D PDF.

additional 10 min. The number of insertion attempts was recorded for all groups as the number of times the male copulatory organ reached the female genitalia without coupling to the female's genital opening. Except for the group in which only one insertion was allowed, the number of successive insertions with alternating pedipalps was recorded. After copulation, the status of the scapus was determined under the stereo microscope.

Data Analysis

All the tests were performed in R [35]. The number of insertions was non-normally distributed and is given as median, IQR, and range. Differences in proportions were tested using chi-square or Fisher's exact test. The copulation success of mutilated females from undisturbed mating trials, intact females from single-insertion matings, and experimentally mutilated females were compared using pairwise Fisher's exact test with Bonferroni correction with the package *fmsb* [36].

Literature Survey

In order to assess the prevalence of external female genital mutilation in spiders, we scrutinized the spider taxonomic literature for notes, descriptions, and drawings of potential cases of genital mutilation. This amounted to a conservative estimate of 80 additional species with external mutilation of female genitalia.

SUPPLEMENTAL INFORMATION

Supplemental Information includes one table and an interactive 3D PDF and can be found with this article online at <http://dx.doi.org/10.1016/j.cub.2015.09.074>.

AUTHOR CONTRIBUTIONS

G.U. designed the study and compiled the information on potential spider species with genital mutilation given in [Table S1](#). P.M., J.P., and U.T. conducted the field study. P.M. conducted the laboratory study and analyzed the data. P.M., G.U., and P.S. performed the cryo-fixations and micro-CT reconstruction. P.M. and G.U. wrote the manuscript. J.K. provided essential logistic support.

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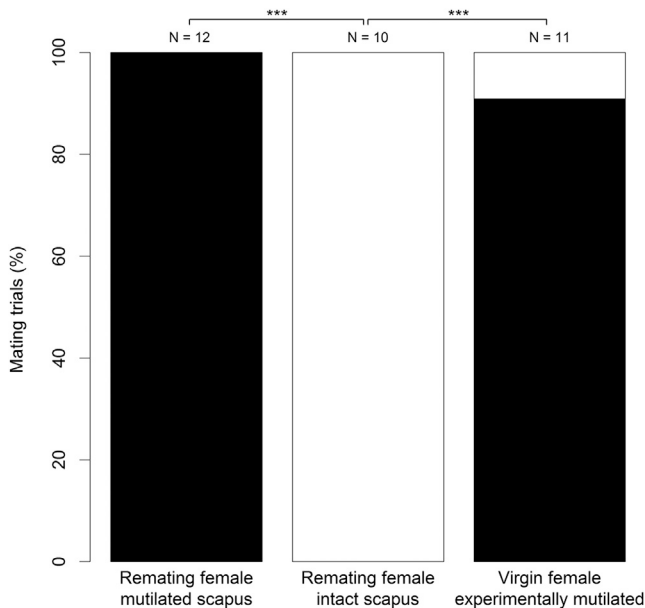


Figure 4. Mutilation as a Means to Impede Subsequent Copulations by Rival Males

Percentages of successful mating (white) and unsuccessful mating (black) of females remating with mutilated or intact scapus from previous matings and virgin females whose scapus was experimentally mutilated (** $p < 0.001$, pairwise Fisher's exact test with Bonferroni correction).

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Current Biology

Supplemental Information

Securing Paternity by Mutilating

Female Genitalia in Spiders

Pierick Mouginot, Josepha Prügel, Ulrike Thom, Philip O.M. Steinhoff, Janusz Kupryjanowicz, and Gabriele Uhl

Table S1, related to Figure 1. Reported cases of potential external female genital mutilation in spiders, collected from a non-exhaustive search through taxonomic literature.

Family	Species	Structure damaged	References
Araneidae	<i>Acroaspis</i> sp.(several species of this genus)	Scapus	V. Framenau 2015, pers. comm.
Araneidae	<i>Aculepeira armida</i>	Scapus	S1
Araneidae	<i>Aculepeira carbonaria</i>	Scapus	S1
Araneidae	<i>Aculepeira carbonarioides</i>	Scapus	S1
Araneidae	<i>Aculepeira ceropegia</i>	Scapus	S1, S2, S3, S4
Araneidae	<i>Aculepeira packardi</i>	Scapus	S1
Araneidae	<i>Araneus allani</i>	Scapus	S5
Araneidae	<i>Araneus corticarius</i>	Scapus	S6
Araneidae	<i>Araneus groenlandicola</i>	Scapus	S6
Araneidae	<i>Araneus hoshi</i>	Scapus	S7
Araneidae	<i>Araneus nordmanni</i>	Scapus	Y. Marusik 2008, pers. comm.
Araneidae	<i>Araneus pratensis</i>	Scapus	S5
Araneidae	<i>Araneus pinguis</i>	Scapus	Y. Marusik 2008, pers. comm.
Araneidae	<i>Araneus quadratus</i>	Scapus	S4
Araneidae	<i>Araneus schrencki</i>	Scapus	Y. Marusik 2015, pers. comm.
Araneidae	<i>Araneus tartaricus</i>	Scapus	Y. Marusik 2015, pers. comm.
Araneidae	<i>Araneus tiganus</i>	Scapus	S8
Araneidae	<i>Araneus yukon</i>	Scapus	S4
Araneidae	<i>Araniella displicata</i>	Scapus	S9
Araneidae	<i>Caerostris indica</i>	Ridges	S10
Araneidae	<i>Cyclosa albopunctata</i>	Scapus	S11
Araneidae	<i>Cyclosa argenteoalba</i>	Scapus	S12
Araneidae	<i>Cyclosa atrata</i>	Scapus	S12
Araneidae	<i>Cyclosa confusa</i>	Scapus	S12
Araneidae	<i>Cyclosa ginnaga</i>	Scapus	S12
Araneidae	<i>Cyclosa hamulata</i>	Scapus	S12
Araneidae	<i>Cyclosa insulana</i>	Scapus	S11
Araneidae	<i>Cyclosa japonica</i>	Scapus	S12
Araneidae	<i>Cyclosa kumadai</i>	Scapus	S12
Araneidae	<i>Cyclosa maritime</i>	Scapus	S12
Araneidae	<i>Cyclosa mulmeinensis</i>	Scapus	S12
Araneidae	<i>Cyclosa okumae</i>	Scapus	S12
Araneidae	<i>Cyclosa omonaga</i>	Scapus	S12
Araneidae	<i>Cyclosa vallata</i>	Scapus	S12
Araneidae	<i>Eriophora edax</i>	Scapus	S13
Araneidae	<i>Eriophora fuliginea</i>	Scapus	S13
Araneidae	<i>Eriophora ravilla</i>	Scapus	S13
Araneidae	<i>Gibbaranea gibbosa</i>	Scapus	S3
Araneidae	<i>Larinia bonneti</i>	Scapus	S4, S14
Araneidae	<i>Larinia chloris</i>	Scapus	S15
Araneidae	<i>Larinia elegans</i>	Scapus	S4, S14
Araneidae	<i>Larinia jeskovi</i>	Scapus	S14
Araneidae	<i>Larinia lineata</i>	Scapus	S16, S17
Araneidae	<i>Larinia phthisica</i>	Scapus	S11, S18
Araneidae	<i>Larinia pubiventris</i>	Scapus	S4
Araneidae	<i>Larinia tábida</i>	Scapus	S11, S16, S18

Araneidae	<i>Larinioides cornutus</i>	Scapus	S19
Araneidae	<i>Larinioides patagiatus</i>	Scapus	Y. Marusik 2015, pers. comm.
Araneidae	<i>Larinopa fusiformis</i>	Scapus	S16
Araneidae	<i>Mangora fornicata</i>	Edge of epigynum	S20
Araneidae	<i>Mangora itza</i>	Scapus	S21
Araneidae	<i>Mangora mathani</i>	Edge of epigynum	S20
Araneidae	<i>Metazygia amalla</i>	Base of epigynum	S22
Araneidae	<i>Metazygia castaneoscutata</i>	Scapus	S22
Araneidae	<i>Metazygia crewi</i>	Scapus	S22
Araneidae	<i>Metazygia limonal</i>	Scapus	S22
Araneidae	<i>Metazygia mundulella</i>	Base of epigynum	S22
Araneidae	<i>Metazygia saturnino</i>	Base of epigynum	S22
Araneidae	<i>Metazygia viriosa</i>	Scapus	S22
Araneidae	<i>Metazygia voluptifica</i>	Scapus	S22
Araneidae	<i>Novakiella trituberculata</i>	Scapus	V. Framenau 2015, pers. comm.
Araneidae	<i>Ocrepeira abiseo</i>	Scapus	S23
Araneidae	<i>Ocrepeira fiebrigi</i>	Scapus	S23
Araneidae	<i>Ocrepeira malleri</i>	Scapus	S23
Araneidae	<i>Parawixia kochi</i>	Scapus	V. Framenau 2015, pers. comm.
Araneidae	<i>Plebs arleneae</i>	Scapus	S24
Araneidae	<i>Plebs patricius</i>	Scapus	S24
Araneidae	<i>Siwa atomaria</i>	Scapus	Y. Marusik 2006, pers. comm.
Linyphiidae	<i>Bolyphantes punctulatus</i>	Scapus	S25
Lycosidae	<i>Pardosa lapponica</i>	Upper pocket	Y. Marusik 2008, pers. comm.
Oxyopidae	<i>Oxyopes heterophthalmus</i>	Scapus	Y. Marusik 2008, pers. comm.
Oxyopidae	<i>Oxyopes licenti</i>	Scapus	S26
Oxyopidae	<i>Oxyopes ramosus</i>	Scapus	Y. Marusik 2008, pers. comm.
Stiphidiidae	<i>Elleguna major</i>	Lateral margins	S27
Stiphidiidae	<i>Elleguna minor</i>	Median septum	S27
Stiphidiidae	<i>Jamberoo johnnoblei</i>	Knob	S27
Stiphidiidae	<i>Karriella treenensis</i>	Knob	S27
Tetragnathidae	<i>Guizygiella guangxiensis</i>	Scapus	S28
Theridiidae	<i>Faiditus arthuri</i>	Hood	S29
Theridiidae	<i>Euryopsis quinqueguttata</i>	Scapus	S30

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3.2. Chapter 2: Evolution of external female genital mutilation: why do males harm their mates?

Pierick Mouginot¹, Gabriele Uhl¹ and Lutz Fromhage²

¹General and Systematic Zoology, Zoological Institute and Museum, University of Greifswald, Greifswald 17489, Germany

²Department of Biological and Environmental Science, University of Jyväskylä, PO Box 35, Jyväskylä 40014, Finland

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Author for correspondence:

Pierick Mougnot

e-mail: pierick.mougnot@gmail.com

Evolution of external female genital mutilation: why do males harm their mates?

Pierick Mougnot¹, Gabriele Uhl¹ and Lutz Fromhage²

¹General and Systematic Zoology, Zoological Institute and Museum, University of Greifswald, Greifswald 17489, Germany

²Department of Biological and Environmental Science, University of Jyväskylä, PO Box 35, Jyväskylä 40014, Finland

PM, 0000-0003-2967-065X; LF, 0000-0001-5560-6673

Sperm competition may select for male reproductive traits that influence female mating or oviposition rate. These traits may induce fitness costs to the female; however, they may be costly for the males as well as any decrease in female fitness also affects male fitness. Male adaptations to sperm competition manipulate females by altering not only female behaviour or physiology, but also female morphology. In orb-weaving spiders, mating may entail mutilation of external structures of the female genitalia, which prevents genital coupling with subsequent males. Here, we present a game theoretical model showing that external female genital mutilation is favoured even under relatively high costs of mutilation, and that it is favoured by a high number of mate encounters per female and last-male sperm precedence.

1. Introduction

Males may evolve traits that shift the remating or oviposition rate of the female from the female's optimum towards their own due to selection on competitive fertilization success [1,2]. Defensive adaptations to sperm competition include mate guarding, copulatory plugs, manipulative seminal fluids and internal genital damage. These adaptations can manipulate the female by altering her behaviour or physiology, but also her genital anatomy [3]. Examples of male-inflicted damages to female genitalia have been documented in numerous taxa, especially among arthropods [4,5]. Most of these genital damages are inflicted internally by the male intromittent organ, and it is debated how these harmful traits have evolved [6–8].

Recently, it has been described that males inflict external damage to the female genitalia in two species of orb-weaving spiders as a defensive adaptation to sperm competition [9,10]. In the course of copulation, males mutilate an external genital structure of the female that is necessary for genital coupling. The lack of this structure prevents the female from

remating [9]. Although probably widespread among spiders [9], the selection regime for external female genital mutilation (EFGM) is difficult to understand, as any decrease in female fitness caused by the mutilation should also reduce the male's fitness [1]. Here, we present a game theoretical model to explore the conditions under which EFGM can evolve and be maintained.

2. Model and results

We consider an infinite population with a sex ratio at maturity of r males per female. During a mating season, each female experiences a number n of mate encounters with different males. In the absence of genital mutilation, females mate with every male they encounter. After that, the female lays eggs and dies. For simplicity, n does not vary between females. Because every mate encounter involves a male and a female, the total number of encounters must be the same for both sexes, implying that each male encounters on average n/r females. We consider two mating strategies for males: 'harmless' males do not perform mutilation of the female genitalia during copulation, nor do they prevent the females from remating. 'Mutilator' males damage every female they mate with. This reduces female fitness by proportion α (the cost of mutilation) and prevents females from remating with subsequent males. Following a game theoretic approach [11], we seek conditions where each strategy can invade (i.e. is favoured by selection when rare) and is an evolutionarily stable strategy (ESS; a strategy which, when common, cannot be invaded by the alternative strategy). For this purpose, we compare the fitness of a rare 'mutant' strategy to the fitness of the 'resident' strategy adopted by the majority of the population. We consider the case where *mutilator* is the mutant strategy and *harmless* is the resident strategy, and vice versa. In doing so, we follow the standard assumption that the mutant strategy is so rare that its effect on the resident strategy's fitness is negligible. See table 1 for a summary of the variables.

2.1. Each female encounters only a single male

We begin by establishing a general result that holds regardless of patterns of sperm precedence. Consider the case where each female encounters only a single male ($n = 1$) and consequently each encounter leads to 100% paternity. Defining the fitness of an unmutilated female as 1, and expressing male fitness in relation to this, the fitness W_H of a *harmless* male is then equivalent to his number of mate encounters, n/r

$$W_H = \frac{n}{r} \frac{1}{n} = \frac{1}{r}. \quad (2.1)$$

Compared to this, the fitness W_M of a *mutilator* is reduced by the cost of mutilation (α)

$$W_M = \frac{n}{r} \frac{1}{n} (1 - \alpha) = \frac{1}{r} (1 - \alpha). \quad (2.2)$$

It is a special property of the $n = 1$ case that the fitnesses of both strategies do not depend on which strategy is currently rare or common in the population. Thus, substituting equations (2.1) and (2.2) into $W_H > W_M$, we obtain

$$\alpha > 0, \quad (2.3)$$

as the condition where *harmless* males have higher fitness than *mutilators* at any frequency. This means that in the $n = 1$ case, mutilation is selected against whenever it imposes any cost on females.

2.2. Each female encounters more than one male

2.2.1. Mutilator invasion

We consider the case where each female encounters more than one male ($n > 1$) and the resident strategy is *harmless*. As the female will mate with all n males she encounters, the paternity over a female's offspring will be shared among her mates. Here, we consider the possibility that either the first or the last male may enjoy an advantage in sperm competition. A *harmless* male has a probability $1/n$ of obtaining the position in a female's mating sequence that grants him sperm precedence, providing him with the paternity share

$$p[i] = \frac{L}{(L + i - 1)}, \quad (2.4)$$

when mating with a female that mates i times in total. This formulation is called a 'loaded raffle' [12], in which the priority male's sperm has L times higher competitive weight than his competitors' sperm.

Table 1. Summary of the model variables.

variable	meaning	constraint
r	sex ratio at maturity	
n	number of mate encounters per female (with different males)	
α	cost of mutilation as a proportion of female fitness	$0 \leq \alpha \leq 1$
i	male's position in the female mating sequence	
L	'loading factor'; characterizes the sperm precedence strength. Sperm precedence is absent if $L = 1$	$L \geq 1$
p	paternity share	$0 \leq p \leq 1$

Parameter L , called a 'loading factor', characterizes the sperm precedence strength and satisfies $L \geq 1$. The absence of sperm precedence (also called a 'fair raffle' process) is included in the formulation as the special case, where $L = 1$.

The paternity of a *harmless* male is then $p[n]$, because the female will mate with all n males she encounters. In every encounter, he also has a probability $(n - 1)/n$ of not obtaining sperm precedence, instead being one of the $n - 1$ males that share the remaining paternity, $1 - p[n]$. The fitness W_H of a *harmless* resident male is therefore:

$$W_H = \frac{n}{r} \left(\frac{1}{n} p[n] + \frac{n-1}{n} \left(\frac{1-p[n]}{n-1} \right) \right) = \frac{1}{r}. \quad (2.5)$$

While this is independent of $p[n]$, and hence of sperm precedence, sperm precedence becomes important when calculating the fitness of *mutilator* mutants in this population.

2.2.1.1. First-male precedence

In each of his n/r mate encounters, a *mutilator* mutant has a probability $1/n$ of being the i th male to encounter (and mate with) a given female. In each of his matings, a *mutilator* prevents the female from remating with subsequent males. Therefore, as the first male, he gets 100% of the paternity. And as the i th male (where $i > 1$), he limits the female to i matings in total, and is hence one of $i - 1$ males that share the paternity $(1 - p[i])$ left over by the first male. The fitness of a *mutilator* mutant is therefore:

$$W_M = \frac{n}{r} \frac{1}{n} \left(1 + \sum_{i=2}^n \frac{1-p[i]}{i-1} \right) (1-\alpha). \quad (2.6)$$

Substituting the fitness (equations (2.5) and (2.6)) and paternity share (equation (2.4)) equations into $W_M > W_H$, we obtain

$$(1-\alpha) \left(1 + \sum_{i=2}^n \frac{1}{L+i-1} \right) > 1, \quad (2.7)$$

as the condition for which the *mutilator* strategy can invade. This corresponds to the area of parameter space illustrated in figure 1a.

2.2.1.2. Last-male precedence

In each of his matings, a *mutilator* mutant will be the last of the female's mates, thus securing the position that grants him the last-male sperm competition advantage. In each of his n/r mate encounters, a *mutilator* mutant has a probability $1/n$ of being the i th male to encounter (and mate with) a given female. In any case, he will be the last of the female's mates, thus, securing the position that grants him the last-male sperm competition advantage, he will receive paternity $p[i]$. The fitness of a *mutilator* mutant is therefore:

$$W_M = \frac{n}{r} \frac{1}{n} \sum_{i=1}^n p[i] (1-\alpha). \quad (2.8)$$

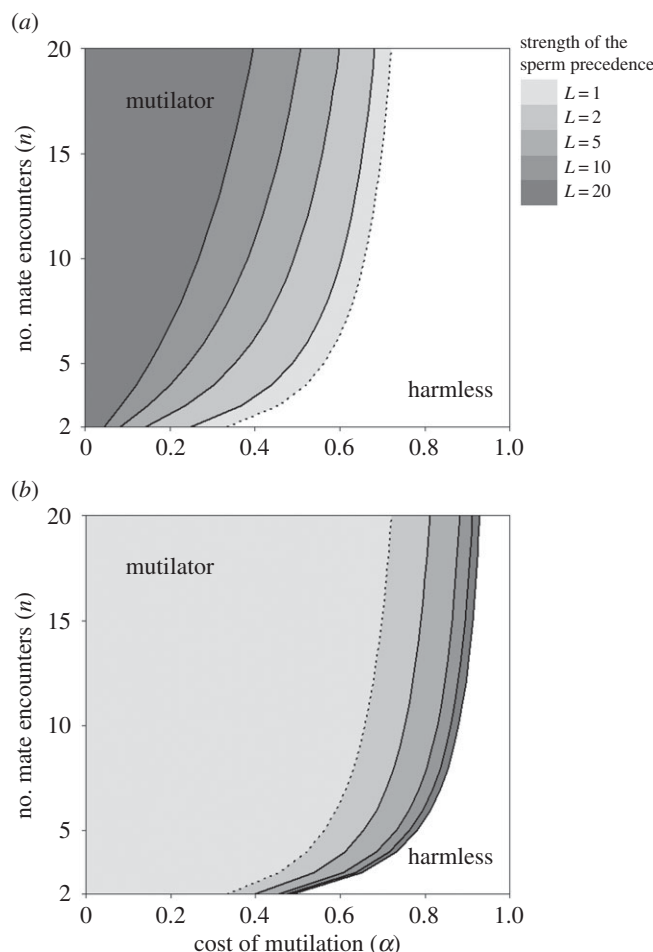


Figure 1. ESS regions under first-male (a) and last-male (b) sperm precedence in parameter space of the cost of mutilation (α) and the number of mate encounters per female (n). The curves represent the limits of the ESS regions for different sperm precedence strengths: $L = 1, 2, 5, 10, 20$, where $L = 1$ (dashed line) represents the situation with no sperm precedence. To the right of each curve, the *harmless* strategy is an ESS. To the left of each curve, the *mutilator* strategy can invade, and is then also an ESS. (a) The ESS region reduces towards darker shaded areas when the strength of the sperm precedence increases. (b) The ESS region extends towards darker shaded areas when the strength of the sperm precedence increases.

Substituting the fitness (equations (2.5) and (2.8)) and paternity share (equation (2.4)) equations into $W_M > W_H$, we obtain

$$(1 - \alpha) \sum_{i=1}^n \frac{L}{L + i - 1} > 1, \quad (2.9)$$

as the condition where the *mutilator* strategy can invade. This corresponds to the area of parameter space illustrated in figure 1b.

2.3. Mutilator stability

We now consider the case where each female encounters more than one male ($n > 1$) and *mutilator* is the resident strategy. In this situation, females are mutilated at their first mating and *mutilators* obtain full paternity. Therefore, a male only gets any paternity when he encounters a virgin female, as happens in $1/n$ of his encounters. If a *harmless* male enters the population of *mutilator* males and mates with a virgin female, the female can still suffer the cost of mutilation from a subsequent mating with a *mutilator*. The fitness of a *harmless* mutant is thus

$$W_H = \frac{n}{r} \frac{1}{n} p(1 - \alpha) = \frac{p(1 - \alpha)}{r}, \quad (2.10)$$

where p is the *harmless* mutant's paternity share. The cost of mutilation is present in the fitness expression of W_H (equation (2.10)) because of the subsequent mating by a *mutilator*. By contrast, the fitness of a resident *mutilator* is

$$W_M = \frac{n}{r} \frac{1}{n} (1 - \alpha) = \frac{1 - \alpha}{r}, \quad (2.11)$$

because *mutilators* obtain full paternity when mating with virgin females. Thus, substituting the fitness equations (equations (2.10) and (2.11)) into $W_M > W_H$, we obtain

$$p < 1, \quad (2.12)$$

as the condition where *mutilator* is an ESS. Therefore, *mutilator* is an ESS as long as the *harmless* mutant does not obtain full paternity (i.e. $p < 1$). This is true whenever a subsequent mating reduces the first male's paternity below 100%, regardless of n and the exact pattern of sperm precedence. This includes, but goes far beyond, all the conditions where the *mutilator* strategy can invade (see above). In other words, the conditions under which *mutilator* is stable are much broader than the conditions under which it can invade.

3. Discussion

Our model predicts that the evolution of EFGM can evolve even under relatively high costs of mutilation (α), and that it is favoured by a high number of mate encounters per female (n) and ancestral last-male sperm precedence (figure 1a,b).

The cost of mutilation is an assumption of the model. It characterizes any cost that is possibly incurred after a physical damage as wound healing, increased immune response or infection risk [13–16]. Although there is currently no evidence for EFGM reducing female fitness, this absence of costs may be the result of selection on females for reducing such costs [1,2,17]. However, when it first evolved, EFGM was probably costly because females had not yet evolved any counteradaptation. The role of mutilation costs in this context is straightforward: for any given paternity share that a *mutilator* might attain through his matings, his resultant number of offspring is proportional to the number of offspring produced by his mates. Thus, other things being equal, increasing the mutilation cost (α) reduces the extent to which *mutilators* can benefit from their behaviour, up to the point of making it impossible for them to invade. Even though the prediction is that a strategy is more likely to evolve for low costs, EFGM is still beneficial up to relatively high costs (figure 1a,b). The limiting aspect of mutilation costs concurs with the theory predicting that harmful males can be favoured provided that the benefits from harming their mates outweigh the costs of reducing their mate's offspring production [1]. Once *mutilators* are common, however, and all females are mutilated sooner or later (because $n > 1$), then mutilation costs no longer reduce the fitness of *mutilators* compared with *harmless* males. This explains why the *mutilator* strategy is stable under much broader conditions than those that allow its invasion.

Taken together, the result that EFGM is stable once it evolves, and the conjecture that mutilation costs are reduced over evolutionary time, leads to another prediction: regardless of current mutilation costs, we expect EFGM to occur more frequently in species in which EFGM invasion would have been possible even under high ancestral costs of mutilation.

Our model shows the evolution of EFGM to be facilitated by a high number of mate encounters per female (n) (figure 1a,b). This is because the mutilation increases a *mutilator's* paternity only in situations where the female will encounter at least one subsequent male in the future, who then cannot mate as a result of the mutilation. By contrast, if a *mutilator* is anyway the last male to encounter a given female, then the mutilation merely causes unnecessary damage. This maladaptive outcome occurs in a proportion $1/n$ of mate encounters, and hence becomes more likely if n is small. It is worth noting that it does not matter for this argument if males are monogamous or polygynous. For example, $n = 2$ could apply if the sex ratio is even and males mate twice, or if the sex ratio is male-biased and males mate only once.

Harmful adaptation to sperm competition should be more common in populations with last-male sperm precedence because the risk of a decreased sperm competition success due to the female remating amplifies the advantage of securing the female [18]. Indeed, EFGM invades more easily if the sperm precedence pattern is last-male precedence (figure 1b). This occurs because *mutilators* always secure the position of 'last male', which is particularly advantageous under last-male sperm precedence. By contrast, first-male precedence hinders *mutilator* invasion (figure 1a) because preventing female remating

is less advantageous if subsequent males obtain little paternity. We predict that EFGM occurrence across spider groups is associated with current or ancestral last-male sperm precedence.

While our predictions do not explicitly depend on the sex ratio at maturation (r), it would be misleading to conclude from this that r is irrelevant in the context of EFGM. In fact, our model merely predicts that r has no additional (independent) effect for a given number of mate encounters per female (n). This does not rule out the possibility that the sex ratio has an effect *via* the number of mate encounters. Indeed, other things being equal, increasing the number of males per female should also increase the mate encounters per female. However, mate encounters may also depend on many other factors, including population density, habitat structure, movement ability, male mortality during mate search, male sperm limitation, as well as the timing of maturation. While we did not model these factors explicitly, they are implicitly accounted for insofar as they affect the number of mate encounters per female (n).

Once EFGM has evolved, it is likely to have further evolutionary consequences that are not captured by our model. For example, as EFGM makes it beneficial to mate with virgin females, it may select for protandry (i.e. males maturing before females). Interestingly, because protandry is generally expected in species with first-male precedence [19], but (ancestral) first-male precedence hinders the evolution of EFGM (figure 1*a*), this suggests a particular sequence of evolutionary events: if protandry is found in species with EFGM, then our model suggests that EFGM evolved before protandry. However, it is also possible that protandry increases the sex ratio at maturation, which then increases the mate encounters per female, thereby facilitating the evolution of EFGM (figure 1*a,b*). Either way, we would empirically expect to find a positive association between EFGM and protandry.

While our present model has focused on the evolution of male behaviour, EFGM should also select for evolutionary responses in females. There are two main ways in which females could adapt to EFGM: resistance or tolerance [1,2,17,20,21]. By evolving resistance traits such as mutilation avoidance behaviour or more sclerotized genitalia, females may avoid EFGM along with the associated costs. Alternatively, females may evolve tolerance traits (or ‘palliative adaptations’ [17]), such as modified genitalia, that reduce (and eventually eliminate) the costs of mutilation, without preventing EFGM as such. While it seems difficult to predict which of these pathways is more likely to occur, the pathway taken should affect the probability that EFGM can in fact be observed: if females evolve resistance, this will tend to eliminate the evidence that EFGM ever existed. By contrast, if females evolve to minimize the associated costs, EFGM may readily be observed in the long run. Another evolutionary response that is worthy of investigation is how EFGM affects the evolution of female mate choice. However, as our focus here has been on male decisions, it is beyond the scope of our study.

Data accessibility. As this is a theoretical work, this does not apply to our study.

Authors’ contribution. P.M. and L.F. contributed through modelling and writing. G.U. contributed through discussions and writing.

Competing interests. We have no competing interests.

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3.3. Chapter 3: Differential oxidative costs of bodily harm and genital damage in an orb-weaving spider

Pierick Mouginot¹, Gabriele Uhl¹ Nia Toshkova², and Michaël Beaulieu^{1,3}

¹ Zoological Institute and Museum, University of Greifswald, Greifswald 17489, Germany.

² National Museum of Natural History, Sofia 1000, Bulgaria

³ German Oceanographic Museum, Stralsund 18439, Germany

In preparation for submission

Title: Differential oxidative costs of bodily harm and genital damage in an orb-weaving spider

Authors: Pierick Mougnot¹, Gabriele Uhl¹, Nia Toshkova², and Michaël Beaulieu^{1,3}

Affiliation:

1 Zoological Institute and Museum, University of Greifswald, Greifswald 17489, Germany

2 National Museum of Natural History, Sofia 1000, Bulgaria

3 German Oceanographic Museum, Stralsund 18439, Germany

Correspondence: pierick.mougnot@gmail.com

Keywords: wound, tissue loss, genital damage, harmful male trait, oxidative status

Abstract:

Tissue loss typically occurs because of sub-lethal predation, abiotic physical damage, and agonistic behaviours between conspecifics. Such loss may be costly when it decreases the overall performance of injured animals by limiting their ability to exploit resources and by affecting their homeostasis. By eliciting a physiological response, such as healing and immune responses against infection, injured animals may minimize these direct costs. However, this physiological response may also limit resources available for reproduction. Here, we investigated the physiological response of female orb-weaving spiders to body and genital damage. For this purpose, we mimicked body and genital damage by experimentally amputating one leg and the external genital structure of females and measured their oxidative status. Leg amputation led to a shift in the oxidative status of female spiders, whereas genital amputation did not. These results were highly robust, as they were consistent across different markers of oxidative damage measured in different individuals in two distinct experiments. Hence, our study provides good evidence that a physical harm inflicted to the locomotory system of female spiders affects their oxidative balance, whereas a damage to their external genitalia does not. Our study is the first to investigate the oxidative costs of tissue loss in a spider species. Our findings suggest that the cost due to genital damage itself is rather a collateral harm and may not fuel the sexual conflict over the mutilation of female genitalia in spiders.

Introduction:

In nature, tissue loss typically occurs because of sub-lethal predation, abiotic physical damage, and agonistic behaviours between conspecifics [1]. Such loss may be costly when it decreases the overall performance of injured animals by limiting their ability to exploit resources (*e.g.* reduced locomotor and feeding ability) and by affecting their homeostasis (*e.g.* because of fluid loss and infection). By eliciting a physiological response, such as healing and immune responses against infection, injured animals may minimize these direct costs. However, investing in such self-maintenance mechanisms may limit resources available for other functions, such as reproduction [2]. This investment trade-off has been suggested to be mediated by variation in oxidative status [3]. For instance, *Bicyclus anynana* butterflies appear to solve the trade-off between fecundity and longevity by increasing antioxidant defences under conditions decreasing fecundity and prolonging lifespan [4]. Indeed, antioxidant defences can neutralize the action of oxidizing species on biomolecules, thereby limiting the generation of oxidative damage in tissues and favouring the survival of the whole organism [3]. However, in the case of a healing and immune response, injured organisms may facilitate these maintenance processes by locally reducing their antioxidant response, as oxidizing species may eliminate pathogens and enhance cell communication during tissue repair [5, 6]. In order to minimize oxidative damage on their own tissues, injured animals may simultaneously reduce their physical activity thereby reducing their overall production of oxidizing molecules [4].

Tissue loss does not only occur because of predation, physical damage, and agonistic behaviours but also during copulation. Indeed, in a broad range of species, males often harm females while transferring sperm and seminal fluids [7] by inflicting physical damage inside or outside females' genitalia [8]. Because sperm and seminal fluids represent resources that females may use, they can affect the physiology of inseminated females [8] and may therefore confound the actual physiological cost of trauma during copulation. In several spider species, males mutilate the outer structures of female genitalia [9, 10], which makes it possible to disentangle the actual costs due to genital damage from other copulation effects. Investigating the effects of male harm on female

oxidative status may therefore further our understanding on females' life-history investment pattern after being mutilated during copulation.

Here, we investigated the female physiological response to body and genital damage by measuring their oxidative status. To mimic body and genital damage, we applied experimental ablation of one leg and of the scapus of females. If males and females coevolved to reduce the physiological costs of genital mutilation [11], we expected leg ablation to be costlier and to trigger more deleterious effects on females' oxidative status than genital mutilation.

Material and Methods

Study animals:

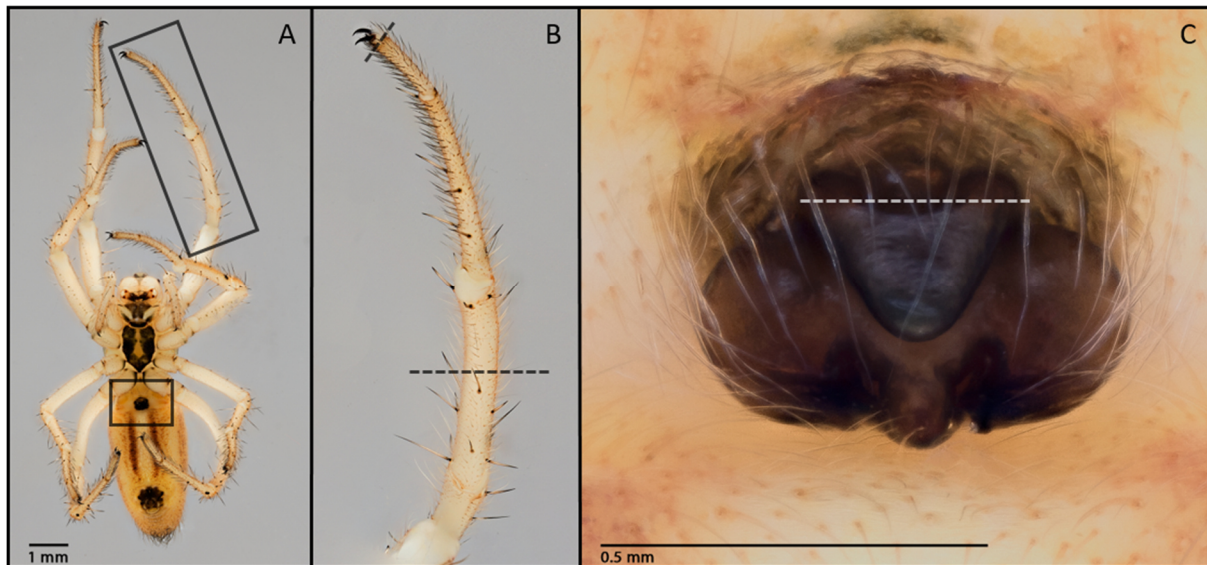
We collected sub-adult (one moulting stage from adulthood) females of the orb-weaving spider *Larinia jeskovi* [12] in August 2015 and 2016 in the Biebrza National Park, Poland (53°21'01.36" N, 22°34'37.45" E). In the laboratory, we housed females individually in 250 mL plastic cups at room temperature and natural light cycle. Females were fed with one fly (*Lucilia sericata*) every three days and watered daily. Sub-adult females (one moulting stage from adulthood) were checked daily for moulting events. After their final moult, adult females were used for the experimental setup.

Experimental setup (figure 1):

In order to assess the effect of tissue loss on females' oxidative status, we experimentally wounded them by amputating one of their fore legs (randomly right or left leg) or manually ablating their scapus [9]. Toward this end, females were first immobilized under a mesh and then wounded under a stereomicroscope with dissection scissors for the leg and forceps for the scapus. A control group was left intact but was similarly handled. In 2015, 30 females were randomly assigned to the three treatment groups (control, tibia-amputated, scapus-amputated). In 2016, we repeated the same procedure with an additional wounding treatment to assess the effect of a leg ablation comparable in terms of tissue loss to the scapus mutilation. In this additional group, one of the fore legs was amputated at 200 µm of its tip. Seventy-two females were randomly assigned to the

four treatments (control, tibia-amputated, leg-tip-amputated, scapus-amputated). Eight hours after treatment, females were cryofixed with liquid nitrogen and stored at -80°C for later analysis.

Figure 1: *Larinia jeskovi* female in ventral view (A), experimental amputation treatments on leg (B) and external female genitalia (C). Dashed lines represent the location of the experimental amputations.



Oxidative stress markers:

Before measurements, all appendages (legs, pedipalps) were removed from the frozen specimens on dry ice, and the body of each female was weighed to the nearest 0.01 mg (Sartorius LE225D; Sartorius AG, Göttingen, Germany) before being mixed with PBS buffer.

In 2015, we measured two markers of the oxidative status of spiders. We used the OXY-absorbent test (Diacron International, Grosseto, Italy) to measure the total antioxidant capacity of samples (expressed in millimole of HOCL neutralized) and the d-ROM test (Diacron International, Grosseto, Italy) to measure the concentration of hydroperoxides, resulting from oxidative damage on organic substrates (expressed in milligrams per decilitre of H_2O_2 equivalent). For both tests, we followed the procedure described in [13]. d-ROM test measurements did not work for three individuals (whose values were below the detection threshold) which were therefore excluded from analyses. This resulted in a sample size of 9 control females, 10 tibia-amputated females, and 8 scapus-amputated females.

In 2016, we measured malondialdehyde (MDA) levels to assess oxidative damage on lipids (expressed in mmol per milligram). The thorax and the abdomen were homogenized together with Triton buffer (7.5 μ l for each 1 mg sample) through high-speed shaking (three times for 1 min; 24 shakes/s). The resulting homogenates were centrifuged at 13,000 rpm for 30 min at 4°C, and the supernatant was then transferred to a new tube and centrifuged again at 13,000 rpm for 15 min at 4°C. The second supernatants were used to analyze total protein concentration and MDA. Lipid peroxidation levels were determined by measuring MDA concentrations using the commercial kit MDA Microplate Assay Kit (Cat. no. CAK1011; Cohesion Bioscience) at 532 nm and 600 nm. One female from the mid tibia amputation group was found dead before cryofixation, and one female was injured during the experimental amputation of the scapus. Both females were excluded from further analyses. Moreover, we were able to measure MDA levels in only 62 females (others showing levels lower than the minimal detection threshold). Thus, 62 females were used for MDA measurements (17 control females, 15 tibia-amputated females, 16 leg-tip-amputated females, and 14 scapus-amputated females).

Total protein concentration of all samples was determined using the Bradford protein assay with microplate reader at 595 nm.

Statistical analyses:

To test the effects of treatment on antioxidant capacity, hydroperoxide and MDA levels, we built linear models with antioxidant capacity, hydroperoxide or MDA levels as dependent variables, and treatment, age, body mass and protein concentration (to correct for concentration differences between samples) as independent variables. We checked linearity assumptions graphically as well as with a Bartlett test for homoscedasticity and Shapiro test for normality of the residuals which, revealed no violation of linearity assumptions for hydroperoxide and MDA levels. The model for antioxidant capacity required the exclusion of an outlier (from control treatment) to match linearity assumptions. There was no significant correlation among the variables (table S1, Suppl. Mat.) leading to no collinearity issues among explanatory variables in our three models. In all models, quantitative variables were centred and standardized [14].

For each model, we considered all plausible candidate models and ranked them according to their AICc value [15, 16]. To evaluate the contribution of each predictor to the model prediction, we calculated its sum of Akaike weights and used “full model averaging” to calculate parameter estimates β [16]. Since the sum of weights may provide a poor evaluation of the predictors’ importance [17], we calculated the 85% confidence interval for each parameter estimate [18]. Parameter estimates whose confidence interval did not include zero were considered as having a significant effect. The evaluation of predictors’ contribution results in parameter estimates for which the first level of a factor is set as a reference. Thus, the results of the amputation treatments are presented as mid-tibia, leg tip and scapus amputation treatments compared to the control treatment as the reference. When body mass was related to oxidative markers (table 1), levels of antioxidant defence markers and oxidative damage were represented after body mass correction. All analyses were performed in R software [19].

Results:

Leg amputation, whether it was applied on the mid tibia or at the leg tip increased antioxidant defences and decreased oxidative damage. Indeed, in 2015, females, whose tibia was amputated, showed higher antioxidant capacity and lower hydroperoxide levels than control females (table 1). Similarly, in 2016, both mid-tibia and leg-tip amputations were associated with decreased MDA levels compared to control females. However, scapus-amputation did not affect the oxidative status of females which, was comparable to that of control females (table 1). Scapus-amputated females did not show any alteration of their oxidative status relative to control females both in 2015 and 2016 (figure 2, table 1). As a result, leg-amputated spiders segregated from other spiders in oxidative space (figure 2).

Figure 2: Levels of antioxidant defences (A: antioxidant capacity) and oxidative damage markers (B: hydroperoxide concentration, and C: MDA concentration) for each experimental amputation treatment in the spider *Larinia jeskovi*. Antioxidant capacity (expressed in per millimole of HOCL neutralized per milligram) was measured with the OXY-adsorbent test, hydroperoxide concentrations (expressed in milligrams per deciliter of H₂O₂ equivalent per milligram) with the d-ROM test. MDA concentrations (expressed in mmol per milligram of proteins per milligram) was used as a marker of oxidative damage on lipids. Levels of antioxidant defences and oxidative damage markers are corrected for body mass.

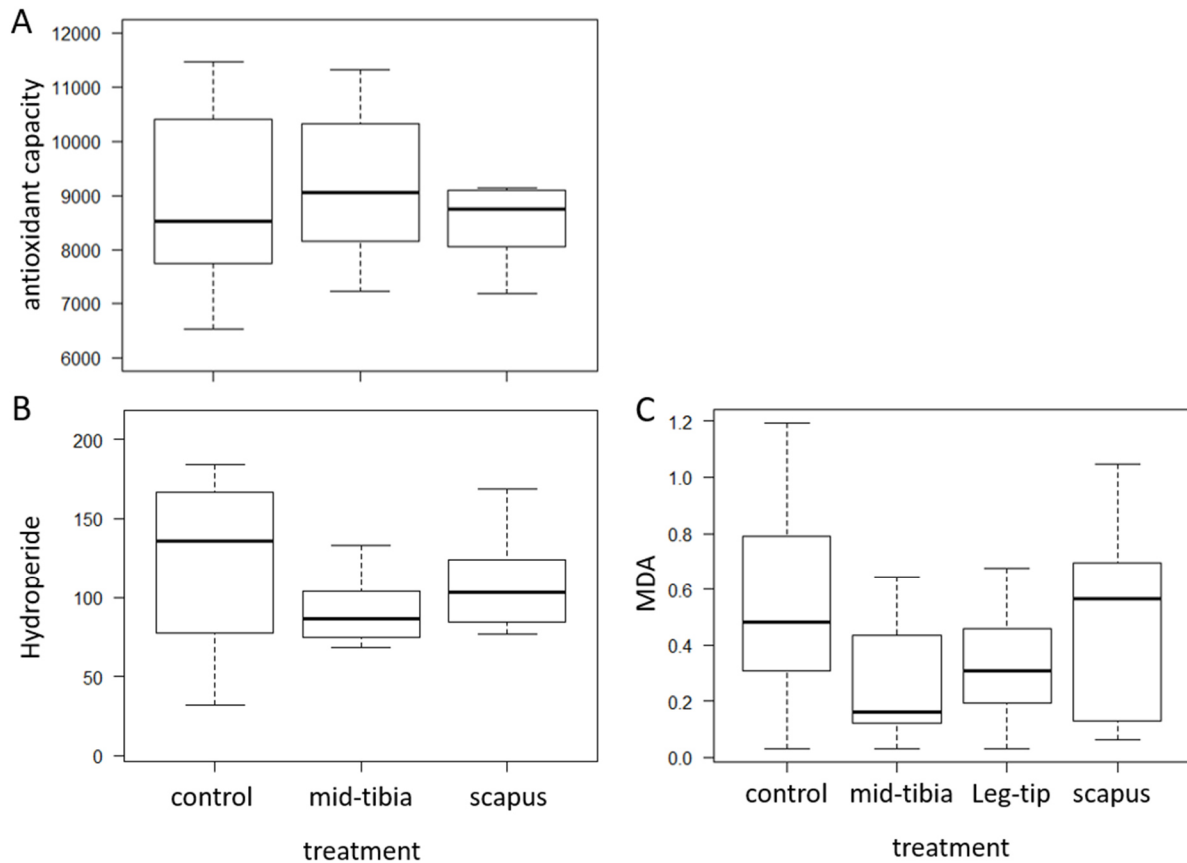


Table 1: Predictor’s sum of weights, parameter significance and 85% confidence interval (CI) after full model averaging on the set of candidate models, assessing the effect of body mass, experimental amputation treatment, age and protein concentration on levels of antioxidant defense (measured by the OXY-adsorbent test) and oxidative damage (measured by the d-ROM test and MDA level) in the spider *Larinia jeskovi*.

Predictor	$\sum w_i$	Parameter	Significance	85% CI
OXY-adsorbent test				
		Intercept	+	278.00; 297.49
Body mass	1	Body mass	+	19.04; 31.54
Protein concentration	0.30	Protein concentration	N.S.	-10.83; 1.81
Treatment	0.22	Mid tibia	+	1.72; 30.29
		Scapus	N.S.	-0.59; 30.71
Age	0.20	Age	N.S.	-4.52; 8.31
d-ROM test				
		Intercept	+	3.24; 4.38
Age	0.58	Age	+	0.09; 0.85
Body mass	1	Body mass	+	1.01; 1.78
Protein concentration	0.23	Protein concentration	N.S.	-0.21; 0.61
Treatment	0.22	Mid tibia	-	-1.93; -0.17
		Scapus	N.S.	-1.68; 0.30
MDA				
		Intercept	+	0.395; 0.634
Body mass	0.24	Body mass	N.S.	-0.066; 0.045
Treatment	0.83	Leg tip	-	-0.370; -0.086
		Mid tibia	-	-0.419; -0.128
		Scapus	N.S.	-0.210; 0.084
Protein concentration	0.24	Protein concentration	N.S.	-0.045; 0.065
Age	0.32	Age	N.S.	-0.088; 0.019

Notes: Parameter estimates after model averaging of treatment (in grey) are compared to the reference level “control”. An estimate whose 85% CI does not include zero is considered significant: N.S. non-significant parameter, + positive significant parameter, – negative significant parameter.

Discussion:

Leg amputation led to a shift in the oxidative status of female spiders irrespective of amputation extent. In contrast, scapus amputation did not affect their oxidative status. These results appear to be highly robust, as they were consistent across different markers of oxidative damage measured in different individuals in two distinct experiments. Hence, our study provides good evidence that a physical harm inflicted to the locomotory system of female spiders affects their oxidative balance, whereas a damage to their external genitalia does not.

In agreement with our predictions, tissue loss due to leg amputation induced a shift in females' oxidative balance, as amputated females showed higher levels of antioxidant defences than intact females. This pattern suggests that amputated females invested in self-maintenance mechanisms by upregulating their production of antioxidant defences. Surprisingly, this upregulation was not associated with stable oxidative damage in amputated females but with lower oxidative damage. Low oxidative damage in amputated females may be due to their lower locomotory activity leading to lower production of oxidizing molecules [4]. Moreover, low physical activity in amputated females may help them to save resources that can be allocated to maintenance mechanisms.

In contrast to leg amputation, genital amputation did not affect females' oxidative balance, as the oxidative status of genitally-damaged females was completely comparable to that of intact females. It might be argued that the extent of the harm was not sufficient enough to induce a detectable physiological response. However, the amputation of the leg tip, an injury comparable to scapus mutilation in terms of tissue loss, affected females' oxidative balance. The absence of effect of scapus amputation on the oxidative status of spiders may be due to the fact that, in contrast to leg amputation, such tissue loss did not have any indirect effects on the overall physical activity of spiders susceptible to alter oxidative markers [4].

External female genital mutilation by males is a common feature of the mating system of several spider species by allowing males to secure paternity [9, 10]. However, the costs and benefits for females are unclear. Our findings suggest that females do not suffer any oxidative costs due genital mutilation, suggesting no costs in terms of self-maintenance in female spiders. As a harmful male adaptation, the mutilation of female genitalia may be subject to a sexual conflict

[7]. However, our study suggests that the cost due to genital damage itself may not fuel this sexual conflict. We argue that the conflict of interests is more likely to lie over female remating. The absence of physiological cost that we observed might therefore be the result of selection on males and females to reduce costs associated to collateral harm that would result in lower reproductive performance in both males and females [8, 11, 20]. This sheds a new light on males' benefits from mutilating female genitalia without impairing female's fecundity and on the evolution of external female genital mutilation in animals [21].

To our knowledge, our study is the first to investigate the oxidative costs of tissue loss in a spider species. Such an approach is highly relevant in the orb-weaving spider *Larinia jeskovi*, where males mutilate females' genital structures during copulation. Moreover, examining the fertility and survival of amputated or mutilated females to assess the costs of tissue loss may be misleading since females may trade-off investment in self-maintenance for a terminal investment in reproduction. This highlights the usefulness of measuring oxidative markers to detect deviation from physiological homeostasis (physiological challenge), and identify life-history investment patterns (physiological response).

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Author contributions:

PM, GU and MB designed the study and contributed through discussions. PM collected animals and carried out the experimental treatments. PM and NT carried out the physiological measurements. PM and MB carried out the statistical analysis. All authors contributed through writing.

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Competing interests:

We have no competing interests.

Ethical statement:

All spiders were handled with care and cold-euthanized.

Supplementary materials S1:

Table 1 (S1): Spearman correlation coefficients between variables used in the analysis of OXY-absorbent and d-ROM tests (A) and Glutathione and MDA levels (B). Significant correlations are indicated in bold (*: p-value < 0.01, **: p-value < 0.01, *** p-value < 0.001).

A	age	Body mass	Protein	d-ROM	OXY-absorbent
age	1				
Body mass	0.063	1		***	***
Protein	0.151	0.226	1		
ROM	0.248	0.747	0.363	1	**
OXY	0.106	0.682	0.033	0.488	1

B	age	Body mass	Protein concentration	MDA
age	1			
Body mass	0.022	1		
Protein concentration	-0.239	0.314	1	
MDA	-0.135	0.071	-0.047	1

Model selection tables:

For each model (tables 2, 3, 4, 5), we calculated the log-likelihood, the AICc, the difference $\Delta AICc$ with the best ranked model (*i.e.* model with the greatest Akaike’s weight), the Akaike’s weight and R^2 . Full model averaged parameters are presented in Table 1.

Table 2 (S1): Model selection table of the candidate linear regressions modelling d-ROM test measurements depending on treatment, age, body mass, and protein concentration.

ROM	Intercept	treatment	age	Body mass	Protein concentration	K	logLik	AICc	Delta	weight	R^2
	+		+	+		4	-43.15	96.12	0	0.375	0.605
	+			+		3	-45.1	97.23	1.111	0.215	0.544
	+		+	+	+	5	-42.98	98.83	2.702	0.097	0.61
	+			+	+	4	-44.54	98.9	2.777	0.094	0.562
	+	+		+		5	-43.06	98.98	2.857	0.09	0.608
	+	+	+	+		6	-41.4	99	2.874	0.089	0.653
	+	+		+	+	6	-42.7	101.6	5.476	0.024	0.618
	+	+	+	+	+	7	-41.29	102.47	6.344	0.016	0.656
	+		+			3	-54.25	115.53	19.411	0	0.101
	+				+	3	-54.37	115.77	19.651	0	0.093
	+					2	-55.69	115.88	19.757	0	0
	+		+		+	4	-53.48	116.77	20.65	0	0.151
	+	+	+			5	-52.83	118.52	22.394	0	0.191
	+	+				4	-54.54	118.9	22.778	0	0.082
	+	+			+	5	-53.26	119.38	23.261	0	0.165
	+	+	+		+	6	-52.09	120.38	24.257	0	0.234

Table 3 (S1): Model selection table of the candidate linear regressions modelling OXY-absorbent test measurements depending on treatment, age, body mass, and protein concentration.

OXY	Intercept	treatment	age	Body mass	Protein concentration	K	logLik	AICc	Delta	weight	R ²
	+			+		3	-114.48	236.04	0	0.423	0.611
	+			+	+	4	-113.82	237.54	1.5	0.2	0.63
	+	+		+		5	-112.63	238.27	2.222	0.139	0.663
	+		+	+		4	-114.44	238.78	2.733	0.108	0.612
	+		+	+	+	5	-113.6	240.21	4.165	0.053	0.636
	+	+		+	+	6	-112.26	240.94	4.894	0.037	0.672
	+	+	+	+		6	-112.36	241.15	5.103	0.033	0.67
	+	+	+	+	+	7	-111.77	243.76	7.716	0.009	0.684
	+					2	-126.76	258.04	21.997	0	0
	+	+				4	-125.12	260.14	24.095	0	0.119
	+		+			3	-126.64	260.37	24.323	0	0.009
	+				+	3	-126.71	260.52	24.474	0	0.004
	+	+	+			5	-124.53	262.06	26.013	0	0.158
	+	+			+	5	-124.91	262.83	26.783	0	0.132
	+		+		+	4	-126.62	263.15	27.11	0	0.01
	+	+	+		+	6	-124.46	265.34	29.301	0	0.162

Table 4 (S1): Model selection table of the candidate linear regressions modelling MDA measurements depending on treatment, age, body mass and protein concentration.

MDA	Int.	treatment	age	body mass	Protein concentration	K	logLik	AICc	Delta	weight	R ²
	+	+				5	-6.25	23.57	0	0.332	0.154
	+	+	+			6	-5.82	25.16	1.586	0.15	0.166
	+	+		+		6	-6.16	25.84	2.272	0.107	0.156
	+	+			+	6	-6.18	25.88	2.307	0.105	0.156
	+					2	-11.43	27.06	3.485	0.058	0
	+	+	+	+		7	-5.76	27.6	4.031	0.044	0.167
	+	+	+		+	7	-5.78	27.63	4.059	0.044	0.167
	+		+			3	-10.72	27.84	4.272	0.039	0.023
	+	+		+	+	7	-6.04	28.15	4.579	0.034	0.16
	+			+		3	-11.42	29.24	5.672	0.019	0
	+				+	3	-11.42	29.26	5.691	0.019	0
	+		+	+		4	-10.68	30.06	6.484	0.013	0.024
	+		+		+	4	-10.69	30.09	6.517	0.013	0.023
	+	+	+	+	+	8	-5.7	30.12	6.547	0.013	0.169
	+			+	+	4	-11.41	31.52	7.951	0.006	0.001
	+		+	+	+	5	-10.64	32.35	8.78	0.004	0.025

3.4. Chapter 4: Female control over mutilation of their genitalia by males

Pierick Mouginot and Gabriele Uhl

General and Systematic Zoology, Zoological Institute and Museum, University of Greifswald,
Greifswald 17489, Germany

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Authors: Pierick Mougnot and Gabriele Uhl

Affiliation:

General and Systematic Zoology, Zoological Institute and Museum, University of Greifswald, Greifswald 17489, Germany.

Correspondence: pierick.mougnot@gmail.com

Abstract:

When females can mate multiply, the interests of both sexes over female remating may not converge, leading to selection for adaptations and counter-adaptations in males and females. In several orb-weaving spiders, males damage external structures of the female genitalia during copulation, which hinders the female from remating. We investigated whether females have control over the mutilation of their genitalia in the orb-weaving spider *Larinia jeskovi*. We found that female sexual cannibalism during copulation reduced the number of insertions a male was able to perform and hence limited the probability of genital mutilation by the male. Genital mutilation did not differ between treatments in which females were subjected to different availabilities of other males before the mating trial: males absent, males in the vicinity of the female ('vicinity'), and males in the female's web ('web'). However, traits of the mating male (size, condition) were significantly correlated with the behavior of the female during mating in 'web' and 'vicinity' treatments. These results suggest that females have control over mutilation by restricting copulation, can respond to the availability of potential mates and can alter the probability of mutilation according to certain male traits. Female sexual cannibalism may represent a counter-adaptation to female genital mutilation and allow choosy females to mate multiply.

Keywords: sperm competition, sexual conflict, external female genital mutilation, orb-weaving spider, female control, sexual cannibalism, female choice

Introduction

Polyandry results in sperm competition and drives the evolution of male traits that increase a male's competitive fertilization success (Parker 1970, Birkhead and Møller 1998, Simmons 2001). In this context, males of many species evolved adaptations that influence sperm use or female remating (Edwards et al. 2015). Male traits that reduce female remating are mate guarding, manipulative seminal fluids, mating plugs and genital damage (Simmons 2014). However, if females benefit from polyandry (Arnqvist and Nilsson 2000, Slatyer et al. 2012), these male adaptations do not coincide with females' interests (Parker 1979, 1984, Arnqvist and Rowe 2005) and may select for traits in females to counteract male manipulations (Arnqvist and Rowe 1995, Elgar et al. 2000).

In a wide range of taxa, males evolved devices that damage females during copulation (Reinhardt et al. 2015). Some of these traits increase the males' success in sperm competition (Hotzy and Arnqvist 2009, Grieshop and Polak 2012, Hotzy et al. 2012). In some spider species, males inflict damages to external structures of the female genitalia that are used for genital coupling, referred to as "external female genital mutilation" (EFGM) (Mouginot et al. 2015). The removal of an external coupling structure hinders the female from remating and thus, secures paternity for the male that performs mutilation (Mouginot et al. 2015, Nakata 2016). However, the physical damage might be costly for the female needing to invest resources in wound healing or in an immune response to prevent infection (Stutt & Siva-Jothy 2001, Morrow & Arnqvist 2003, Otti 2015). Also, EFGM might shift a female's remating rate below its optimum (Arnqvist & Nilsson 2000). Despite negative effects on female fecundity, it was shown in a modelling approach that EFGM can evolve and be maintained (Mouginot et al. 2017).

In orb-weaving spiders, copulation consists of several insertions of the male paired copulatory organ (pedipalps) into paired copulatory openings of the female in order to transfer sperm into the spermathecae (Foelix 2011). In orb-weavers with EFGM, the external genital structure is mechanically cut-off by specific structures of the paired male copulatory organs, the pedipalps (Mouginot et al. 2015). Removal of the female structure requires using both pedipalps in

succession since each can only cut the structure half way. Hence, the number of insertions positively affects the occurrence of genital mutilation (Nakata 2017). Conflict of interests is likely to occur over the number of insertions: males benefit from several copulatory insertions to achieve mutilation, whereas females might benefit from avoiding mutilation to be able to remate. Therefore, control over the number of insertions might be a key aspect for females to counter the severe male manipulation. Sexual cannibalism is a common component of the mating system of spiders and often results in shorter copulation (Schneider 2014). Limiting the number of insertions by fending the male off or attacking him may allow the female to avoid mutilation and to benefit from further mating opportunities with potentially better males (Jennions and Petrie 2000; Bleu et al. 2012). The behavior of the female during mating may depend on whether she perceives additional or alternative potential mating partners: if females can expect more than one mate encounter, they might be choosier and e.g. trade a greater number of insertions with their current mating partner for fewer insertions with several males (Henshaw 2018). Consequently, in species with genital mutilation, females might adjust the number of insertions and their cannibalistic behavior to the perceived mate availability.

In the orb-weaving spider *Larinia jeskovi*, that is best studied for EFGM, the rate of genital mutilation after copulation is very high and cannibalism occurs (Mouginot et al. 2015). We investigated if females have control over copulation and the occurrence of genital mutilation, and if cues of potential mate availability will influence her decision. To this aim, we tested if genital mutilation was less likely when the females had been subjected to cues by other males before contact with the mating partner or not. The treatments differed in the degree of experience with male cues: 1) females had no contact with other males prior to the mating trial (absence), 2) females were subjected to potential chemical cues from other males (vicinity) and 3) females experienced the physical presence of other males on their webs (webs). If females have control over copulation and mutilation and profit from multiple mating, we expected to observe a lower mutilation frequency when other males were perceived either through chemical cues and/or vibration on the web. We further explored whether size, condition and age of both sexes influence the probability of mutilation and assessed whether female choosiness for male traits differed

according to the male cues females experienced. Again, we expected to observe correlations between these traits and the female response in treatments in which other males could be perceived.

Material & Methods

Animal collection

We collected sub-adult (one molting stage from adulthood) males and females and adult males of the orb weaving spider *Larinia jeskovi* (Marusik 1986) in August 2016 in the Biebrza National Park, Poland (53°21'01.36" N, 22°34'37.45" E). In the laboratory, we housed males and females individually in 250 mL plastic cups. The sexes were kept in separate rooms under room temperature and natural light cycle. The spiders were fed with one fly (*Lucilia sericata*) every 3 days and sprayed with water daily. Sub-adults were checked for molting events every day. After their final molt, females were transferred individually to plastic hexagonal boxes (18 × 18 × 6 cm) that were equipped with two lateral, mesh covered windows on opposite sides to provide sufficient air circulation. These boxes, in which all females built webs, were used as the mating arena. Males that performed their final molt in the laboratory were offered to the females as mates. We used the males that were captured as adults only to provide male-presence cues.

Experimental design

Orb-weaving spider females may perceive males by their vibratory signals on the female's web and possibly also by chemical cues released by males (Gaskett 2007, Cross et al. 2009, Uhl and Elias 2011, Scott et al. 2018). 66 females were randomly subjected to one of the three groups that differed in the presence of males before the actual mating trial: i) absence of male (absence group), ii) presence of male in the vicinity of the female's web (vicinity group) and iii) presence of male on the female's web (web group). In the "vicinity group", two males were kept in a plastic box each (5.2 × 5.2 × 3.6 cm) that were glued to the both sides of the mating arena. The boxes of the males were equipped with a window covered with mesh to allow exchange of potential

chemical cues between them and the mating arena. Females in the mating arena were exposed to male vicinity for 24h. In the “web group”, females were subjected to vibratory signals and possibly chemical cues from two successive males wandering on the web. Each male was on the web for 5 minutes with a 1h interval between males. Males moved over the web and courted by vibrating the web but did not achieve physical contact with the female within this time window. The males were removed from the web by an air puff applied directly on the male with a Peleus ball. 31 males that were collected as adults were used as web and vicinity males. Males were used repeatedly but combined in a way that each pair of males was used only once. Before the actual mating trial, the webs of all females were destroyed. Females built new webs that did not contain cues of previous visitors or of males in the vicinity (Uhl and Elias 2011, Scott et al. 2018). Webs of control females without males were also destroyed. The time span between web destruction and the mating trial was 48h.

Mating trials were staged by introducing a randomly selected virgin male into an upper corner of the female’s web. Male *L. jeskovi* perform a courtship behavior typical for araneid spiders (Foelix 2011). In the course of the mating sequence, the male moves about the female’s web, vibrates with the opisthosoma and plucks the threads with his front legs. After achieving physical contact with the female, the male adds a mating thread on female’s web from the female’s position to the edge of the web. The male hangs from the mating thread and plucks it until the female moves onto it. Receptive females approach the male and after foreleg dabbing, they assume copulatory posture. In copulatory posture, foreleg dabbing continues, and the male inserts his paired copulatory organs (pedipalps) one by one into the paired female copulatory openings for sperm transfer. We recorded the number of pedipalp insertions a male achieved. A couple is considered as having copulated when they achieved at least one pedipalp. A mating was considered terminated when the female attacked the male during copulation or when no contact was observed within 5 minutes after the couple had separated. Immediately after mating, we inspected females for mutilation of their external genitalia. The following day, females and males were photographed under a stereomicroscope (Zeiss Discovery V20 Stereo Microscope, Carl Zeiss MicroImaging). The length of the tibia-patella of the first leg as well as width and length of the opisthosoma were measured from the photographs using the software Axiovision 4.8.

Statistical analyses

Irrespective of treatment group, we first investigated the link between occurrence of mutilation and number of insertion, occurrence of mutilation and cannibalism and occurrence of cannibalism and number of insertions for those cases in which copulation occurred (table 1). We tested the correlation between the occurrence of mutilation and number of insertions, and between the occurrence of cannibalism and the number of insertions using separate Spearman's rank correlation tests. The 95% confidence interval of the correlation's coefficient was calculated by bootstrapping 10000 replicates. We compared the number of mutilation occurrences when females cannibalized males and when not with a Fisher's exact test. We further compared the difference in number of insertions between females that cannibalized the male or not using a Wilcoxon rank sum test.

We assessed whether pre-mating male availability, i.e. the treatment groups, affected the occurrence of mutilation, number of insertions and occurrence of cannibalism. We first tested whether the treatments affected the proportion of couples that achieved physical contact (Chi²-test) and assumed copulatory posture (Fisher's exact test) (table 1) to assess differences in receptivity. Then, we compared the proportions of mutilated females after copulation between treatment groups (table 1) with a Fisher's exact test. To test for differences in number of insertions between treatments we used a Kruskal-Wallis test. We compared the occurrence of cannibalism between treatment groups with a Chi²-test.

We conducted an exploratory analysis to identify the male and female traits involved in mutilation occurrence. We then assessed whether female choosiness for male traits differed between treatments by comparing how these traits are correlated to the number of insertions and occurrence of cannibalism for each treatment group. Whether mating trials resulted in copulation and mutilation occurrence may depend on female receptivity or pre-copulatory rejection and also on male's activity or receptivity. Since we aimed to investigate if females controlled copulation and mutilation, we analyzed couples that assumed a copulatory posture (table 1). This way, we avoided analyzing mating trials resulting in no copulation and no mutilation because of male unreceptiveness and focused on female choice when in the copulatory posture.

To explore which female and male traits affected the probability of mutilation, we followed a multi-model inference approach (Burnham & Anderson 2002). When males were cannibalized, it was not always possible to retrieve the male. Thus, the total sample size for size measurements is 38 pairs. The traits investigated are the individual's body size, body condition and age (Supplementary materials, figure S1). Body size was estimated from the tibia-patella length of the first leg (in mm). Body condition was estimated by the residuals of the linear regression of the opisthosoma width against leg length (Jakob et al. 1996; Schulte-Hostedde et al. 2005). Age was calculated as the number of days between the final molt and the mating trial. We built three models that tested three different hypotheses. Model 1 tested for the effect of males' traits on mutilation probability and model 2 tested for the effect of females' traits. Model 3 tested for the effect of the difference in size, condition and age between a mating pair. The difference between female and male condition was calculated as the absolute value of the difference between female and male condition. Each model comprised a logistic regression with the probability of mutilation as the response variable and size, condition and age as model predictors that did not show strong collinearity (Spearman's rank correlation: $-0.4 < \rho < 0.4$). To compare the three hypotheses, we calculated the pairwise evidence ratios of the three full models as the exponential of the log-likelihood of model i divided by exponential of the log-likelihood of model j (Galipaud et al. 2017). For each hypothesis (type of model predictors), we investigated which predictors were influential. For each hypothesis, we considered all plausible candidate models and performed a model selection analysis. For each candidate model, we calculated its Nagelkerke pseudo R^2 (Nagelkerke 1991), its AICc value and its Akaike weight (Burnham and Anderson 2002; Symonds and Moussalli 2011). Models were ranked according their AICc value with models with the lowest AICc value considered the best. To estimate each predictor's contribution, we performed model averaging analyses on each set of candidate models. All predictors were centered and standardized in order to compare their relative contribution on a common scale (Schielzeth 2010). For each predictor, we calculated its sum of Akaike weights and full model averaged parameter estimate β (Symonds and Moussalli 2011) flanked by its 85% confidence interval (Arnold 2010). We interpreted predictors whose confidence interval included zero as uncertain. We then assessed the strength of the correlations between the male traits pointed out by the exploratory analysis and the female

response (number of insertion and cannibalism) for each premating male availability treatment. We assessed correlations by estimating Spearman's rank correlation coefficients. Their 95% confidence interval were calculated by bootstrap (10000 repetitions). A correlation estimate whose 95% CI includes zero was considered uncertain.

All analyses were implemented in the statistical software R v3.4.3 (R Core Team 2017). The MuMIn v1.40.4 (Barton 2018) and RVAideMemoire v0.9-69-3 (Hervé 2018) packages were used for procedures of model selection and model averaging and for computing the confidence interval of the Spearman's rank correlation coefficients by bootstrap, respectively.

Results:

Overall, couples engaged in physical contact in 77.3% of the mating trials (51 of 66 mating trials) (table 1). After physical contact, 92.2% of couples engaged in copulatory posture (47 of 51) (table 1). Among those that assumed the copulatory posture, 27.7% of females were mutilated after copulation (13 of 47 mating trials) and 72.3% cannibalized their mating partner (34 of 47) (table 1). During copulation 0 to 9 insertions were performed (median = 1, IQR = 1-2).

The occurrence of mutilation was positively correlated with the number of insertions: the more insertions, the higher the probability of mutilation (Spearman's rank correlation: $\rho = 0.709$, 95% CI = 0.452, 0.904). The occurrence of mutilation is negatively associated with female cannibalism: only 14.7% (5 of 34) of females that cannibalized their mating partner were mutilated, whereas 88.9% (8 of 9) of females that did not cannibalize their mating partner were mutilated (Fisher's Exact Test: $\chi^2 = 15.216$, $df = 1$, $p\text{-value} < 0.001$, odds ratio = 40.33, 95% CI: 4.13; 2093.46). The occurrence of cannibalism was negatively correlated with the number of insertions (Spearman's rank correlation: $\rho = -0.81$, 95% CI: -0.929; -0.62). Females cannibalized males usually during the 1st insertion (median = 1, IQR = 1-1, min-max: 1-3), whereas surviving males performed a median number of 4 insertions (IQR = 4-4, min-max: 2-9) (Wilcoxon rank sum test, $W = 300$, $p\text{-value} < 0.001$).

Premating male availability treatments affected neither the probability of physical contact between the potential partners (Chi²-test: $\chi^2 = 1.553$, $df = 2$, $P = 0.46$), nor the probability of engaging in copulatory posture (Fisher's exact test: $\chi^2 = 0.711$, $df=2$, $P = 0.82$). The treatments did not differ in the proportions of mutilated females (absence: 33.3%; vicinity: 35.7%; web: 21.4%; Fischer's exact test: $\chi^2 = 0.782$, $df = 2$, $P = 0.67$). The number of insertions during copulation did not differ between treatments (table 1) (Kruskal-Wallis $\chi^2 = 0.567$, $df = 2$, $P = 0.75$). Likewise, the occurrence of cannibalism did not differ between treatments (absence: 80%; vicinity: 71.4%; web: 85.7%; Fischer's exact test: $\chi^2 = 0.875$, $df = 2$, $p = 0.73$).

The exploratory multi-model analysis over all data irrespective of treatment showed that the full models including either male traits, female traits or difference between female-male traits explain 74.8%, 7.9% and 55.9% respectively of the total variance in mutilation probability (table 1). This is congruent with the AICc values of the full and best ranked models and suggests that mutilation probability was not influenced by female traits (table 2). The multi-model analysis showed that several models are supported (table 1). Averaged parameter coefficients for each of the variables are presented in table 3. In the model using male traits, model averaging revealed that mutilation probability is influenced by male size, condition and age. In the trait difference model, only the size difference between the partners seems to influence mutilation probability. According to the evidence ratios (table 4), the full male traits model represents the data better than the female trait and trait difference models. The female traits model has the poorest explanatory power of the three models. Evidence ratios revealed that the full model including male traits explains the data 70 times better than the trait difference full model (table 4). Therefore, further analyses of the mutilation probability were performed using the male traits (size, condition, age). According to the model averaging, male size seems to be the most influential trait (table 3). Male size and age have a positive effect on mutilation probability, i.e. matings with larger and older males resulted in higher mutilation probability, whereas male condition has a negative effect, i.e. matings with male of better condition resulted in lower mutilation probability (table 3, figure 1).

The premating male availability treatments show differences in the correlations between the traits of a female's mating partner (size, condition and age) and the number of insertions or occurrence

of cannibalism. Larger males achieved more insertions in the web group (figure 2A), and they were less likely to be cannibalized in the vicinity and web groups (figure 2B, table S1). This resulted in higher mutilation probability for larger males. Males in better condition achieved less insertions in the web group (figure 2A), and were more likely to be cannibalized in the web group (figure 2B, table S1). This resulted in reduced mutilation probability for males in better condition. Male age did not correlate with the number of insertions or the occurrence of cannibalism in any treatment group (figure 2A, B). A common pattern links male size and body condition since they are both correlated with the number of insertions and cannibalism occurrence only when females were exposed to male presence on female's web before the mating trial (figure 2).

Discussion

Our study indicates that females in the orb-weaving spider *Larinia jeskovi* have control over mutilation of their genitalia by males. Sexual cannibalism employed by the females limits the male to a single insertion, which does not suffice to remove the female genital structure. The premating male availability treatments did not affect the mutilation probability per se, however, an in-depth exploration of traits of both sexes suggests that certain male traits influence female mating behavior particularly under conditions in which other males are perceived.

We predicted that if females profit from multiple mating, they should avoid mutilation when having access to additional potential mating partners. The availability of males before the mating trial did not significantly affect female receptivity, the number of insertions nor the probability of cannibalism and mutilation. One explanation being that females did not perceive the presence of males in the treatments. However, we found that the female's response to specific male traits differed among treatment groups. We assume that the overall effect of mate availability on the occurrence of mutilation was confounded in treatment groups because some male traits had opposite effects on mutilation probability. In addition, the distribution of male trait values was not always homogenous between mate availability treatments (see figure S1).

Male traits had overall more influence than female traits and the difference between female and male traits on the probability of mutilation. Male body size, male body condition and male age

influenced the probability of mutilation significantly, suggesting that females use these traits as cues for mate assessment. Contrary to male size and age, male condition had a negative effect on mutilation probability. This could be explained by the nutritional benefit a male in good condition may represent for a cannibalistic female (Wilder et al. 2009). As a result, females may trade off male condition (nutritional benefits) against male size (genetic benefits). The number of insertions and cannibalism occurrence were correlated with male body size and condition most strongly when females had been exposed to premating presence of males on their web. This is congruent with the expectation that females should be choosier when there are mating opportunities (Kokko & Mappes 2005, Henshaw 2018). Taken together, these results suggest that females can selectively bias the probability of mutilation towards certain males. Moreover, our results suggest that females may perceive the presence of potential mating partners in the environment by vibratory cues produced by males on their webs and by chemical airborne cues of males in the vicinity, however to a lesser degree. Taken together, vibratory and chemical cues might be perceived by females when males are on their web.

Female sexual cannibalism plays an important role in the mating system of several spider species (*e.g.*, species of *Argiope*, *Latrodectus*, Elgar 1992) and has been suggested to be an effective means to control paternity (Andrade 1996, Schneider et al. 2015). Our findings on *Larinia jeskovi* suggest that cannibalism allows females to remate and control the paternity share of the mating partners. Females can avoid or accept mutilation according to certain male traits. Avoiding mutilation by a given male will not only limit the number of sperm transferred due to only a single insertion but also his fertilization success should the female remate with another male. By contrast, accepting mutilation by a particular male grants this male full paternity. If a male is not the first partner, however, but mates with a non-mutilated female, his paternity share can strongly differ depending on how many insertions he is allowed to perform. Although the sperm precedence pattern is unclear in *Larinia jeskovi*, genital mutilation was shown to evolve with higher probability when the sperm of the last male to mate has precedence (Mouginot et al. 2017). By allowing mutilation, a female grants a selected male the last position in the mating sequence, and thus, the largest paternity share. In several spider species, females accept males indiscriminately for the first copulation (Wilder and Rypstra 2007), thereby ensuring a minimal supply of sperm and they

remate only with males of higher quality (trade-up strategy) (Halliday 1983). This may be also the case in *Larinia jeskovi*, since cannibalism occurred mainly at the first insertion, suggesting that females allowed a first insertion without discrimination, but avoided monopolization by their current mating partner. Since females avoid monopolization and discriminate among males during copulation, it is likely that *Larinia jeskovi* females benefit from polyandry (Schneider 2014). Testing mate choice of females that avoided mutilation during their first mating and the effect of male choice on their reproductive success would be desirable.

Sexual cannibalism may function as a counter-adaptation to genital mutilation. However, we cannot rule out that EFGM actually evolved as a male counter-adaptation to female control over paternity by sexual cannibalism, since cannibalism is considered to drive the evolution of a many male counter-adaptations (reviewed in Schneider 2014). Once more information on the distribution of sexual cannibalism and EFGM in spiders is available, plotting the traits on a robust spider phylogeny will help to disentangle adaptation from counter-adaptation and unravel the evolutionary processes that led such adaptations.

Previous observations suggest that in species with EFGM, the rate of mutilation is very high (90%) and the rate of female sexual cannibalism low (3%) (Mouginot et al. 2015, Nakata 2016). However, in our study on *L. jeskovi*, the rate of mutilation is lower (30.2%) and the rate of cannibalism is higher (79.1%). We showed that the rate of mutilation is influenced by the rate of sexual cannibalism through the number of insertions, and that smaller males were more prone to be cannibalized. Males used in the present study on *L. jeskovi* were of a smaller size than males collected during previous mating seasons (Mouginot et al. 2015, unpublished data). This might explain why we observed a lower rate of mutilation and a higher rate of cannibalism.

Our study provides new insights on EFGM and its effect on the mating system. In studies on EFGM, mutilation was considered to impose monogamy on females since females were assumed to have no efficient means to counteract male manipulation and to exhibit no choosiness (Nakata 2016). We showed that females may mate repeatedly and that female cannibalism may counteract male manipulation. The number of insertions necessary for mutilation to occur is a key aspect for females to avoid mutilation and seems to vary within and among species (Mouginot et al. 2015,

Nakata 2016, Nakata 2017). We suggest that investigating the association between the rate of cannibalism and the number of insertions necessary for mutilation occurrence across species that exhibit EFGM may help understanding the coevolution of EFGM and female cannibalism.

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Figure legends:

Figure 1: Mutilation probability (CI 85%) depending on standardized male size (A), male condition (B) and male age (C) predicted from full averaging of the “male traits” model candidates (table 2, 3). Circles are observed data.

Figure 2: Spearman rank’s correlations (95% confidence interval) between the number of insertions (A) or cannibalism occurrence (B) and the standardized male size (green), male condition (orange) and male age (blue) for each pre mating male availability treatment females were exposed to. Correlation coefficients are represented by triangles for male absence, circles for male presence in vicinity and squares for male presence on female’s web. See table S1 for correlation coefficients (95% CI) and sample sizes. A correlation coefficient whose 95% CI includes zero is considered uncertain.

Tables and table legends:

Table 1: Summary of the mating trials with *Larinia jeskovi* females which were exposed to pre-mating absence of males, pre-mating presence of males in female’s web vicinity and pre-mating presence of males on female’s web. For each treatment group, we reported the number of mating trials in which couples achieved physical contact, copulatory posture, copulation (at least 1 copulatory organ insertion). For couples that copulated, we reported the median number of insertions (interquartile range and min-max) and the number of cannibalism and female genital mutilation occurrence.

Premating treatment:			Male absence	Male presence in vicinity	Male presence on female’s web
Total sample size:			22	22	22
Physical contact between male and female:	N = 15	No	3	6	6
	N = 51	Yes	19	16	16
Male and female assumed copulatory posture:	N = 4	No	1	1	2
	N = 47	Yes	18	15	14
Couples that copulated (at least 1 insertion):	N = 4	No	3	1	-
	N = 43	Yes	15	14	14
Number of insertions:	Median:		1	1	1
	IQR:		1-3	1-1.75	1-2
	min-max:		1-6	1-9	1-4
Cannibalism:	N = 9	No	3	4	2
	N = 34	Yes	12	10	12
Mutilation:	N = 30	No	10	9	11
	N = 13	Yes	5	5	3

Table 2: Logistic regressions modelling the probability of mutilation depending on the considered traits: body size, body condition and age; for each predictor: male traits, female traits and traits differences between female and male. For each candidate model, we calculated the log-likelihood, the AICc, the AICc difference with the best ranked model (Delta) according to the model's Akaike weight and the pseudo R².

Predictors	Parameters				K	LogLik	AICc	Delta	Weight	Pseudo R ²
	int	size	condition	age						
male traits										
	int	size	condition	age	4	-7.72	24.66	0	0.612	0.748
	int	size	condition		3	-10.1	26.91	2.251	0.199	0.647
	int	size			2	-12.01	28.35	3.695	0.096	0.557
	int	size		age	3	-10.98	28.66	4.005	0.083	0.607
	int		condition	age	3	-13.5	33.7	9.04	0.007	0.48
	int		condition		2	-15.29	34.91	10.257	0.004	0.379
	int				1	-20.8	43.71	19.056	0	0
	int			age	2	-20.48	45.31	20.647	0	0.025
female traits										
	int				1	-20.8	43.71	0	0.299	0
	int	size			2	-19.9	44.14	0.421	0.242	0.07
	int			age	2	-20.62	45.58	1.863	0.118	0.015
	int		condition		2	-20.65	45.64	1.923	0.114	0.012
	int	size		age	3	-19.79	46.28	2.568	0.083	0.078
	int	size	condition		3	-19.9	46.5	2.783	0.074	0.07
	int		condition	age	3	-20.36	47.43	3.719	0.047	0.034
	int	size	condition	age	4	-19.77	48.76	5.044	0.024	0.079
traits differences										
	int	size			2	-12.69	29.72	0	0.464	0.522
	int	size		age	3	-11.97	30.65	0.93	0.292	0.559
	int	size	condition		3	-12.58	31.86	2.14	0.159	0.528
	int	size	condition	age	4	-11.97	33.15	3.434	0.083	0.559
	int		condition		2	-19.23	42.81	13.087	0.001	0.119
	int				1	-20.8	43.71	13.995	0	0
	int		condition	age	3	-18.83	44.36	14.638	0	0.148
	int			age	2	-20.33	45.01	15.293	0	0.037

"Int" stands for intercept.

Table 3: Full model averaged estimates (85% CI and sum of weights) of logistic regression parameters for each predictor of the probability of mutilation: male traits, female traits and trait difference (female-male).

Predictors	Parameters	Averaged β	85% CI	$\sum w_i$
male traits				
	intercept	- 3.944	- 6.727, - 1.162	
	age	1.318	0.084, 3.674	0.70
	size	3.844	1.204, 6.564	0.99
	condition	- 1.715	- 3.833, - 0.347	0.82
female traits				
	intercept	- 1.207	- 1.788, - 0.625	
	age	- 0.063	- 0.836, 0.370	0.27
	size	- 0.229	- 1.179, 0.098	0.42
	condition	0.039	- 0.433, 0.737	0.26
trait difference				
	intercept	- 2.285	- 3.374, - 1.196	
	age	- 0.268	- 1.663, 0.234	0.38
	size	- 2.848	- 4.428, - 1.276	1.00
	condition	0.048	- 0.742, 1.132	0.24

Averaged parameter estimates are not back-transformed (“logit” link function). A parameter estimate whose 85% CI includes zero is considered uncertain.

Table 4: Comparisons of hypotheses as to which predictors (male traits, female traits or trait difference between female and male) influence the probability of mutilation. Evidence ratios comparing each full model to each other (table 2) were calculated as likelihood of model j divided by likelihood of model i , where j refers to predictors in columns and i to predictors in rows.

	male traits	female traits	trait difference
male traits	1	5.84×10^{-6}	1.426×10^{-2}
female traits	1.711×10^5	1	2.441×10^3
trait difference	70.105	4.097×10^{-4}	1

Log-likelihood values of the full models are shown in table 2.

Figures:

Figure 1:

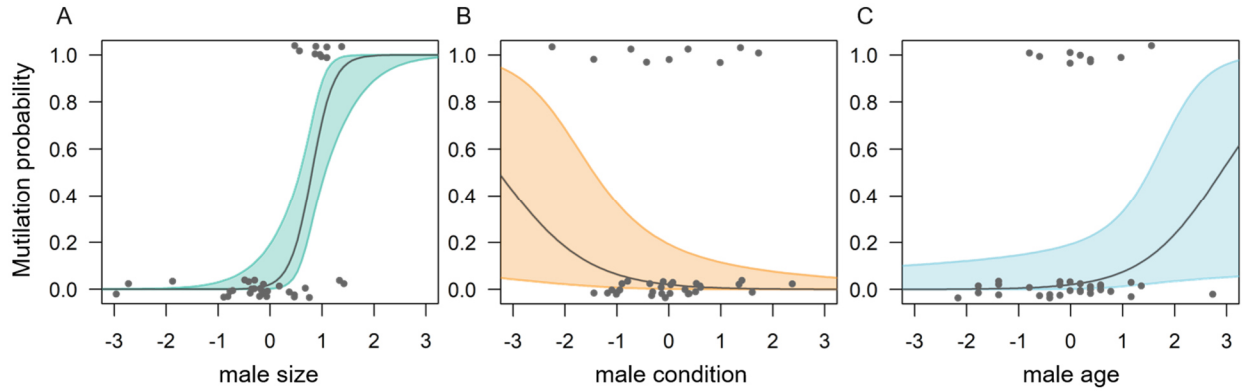
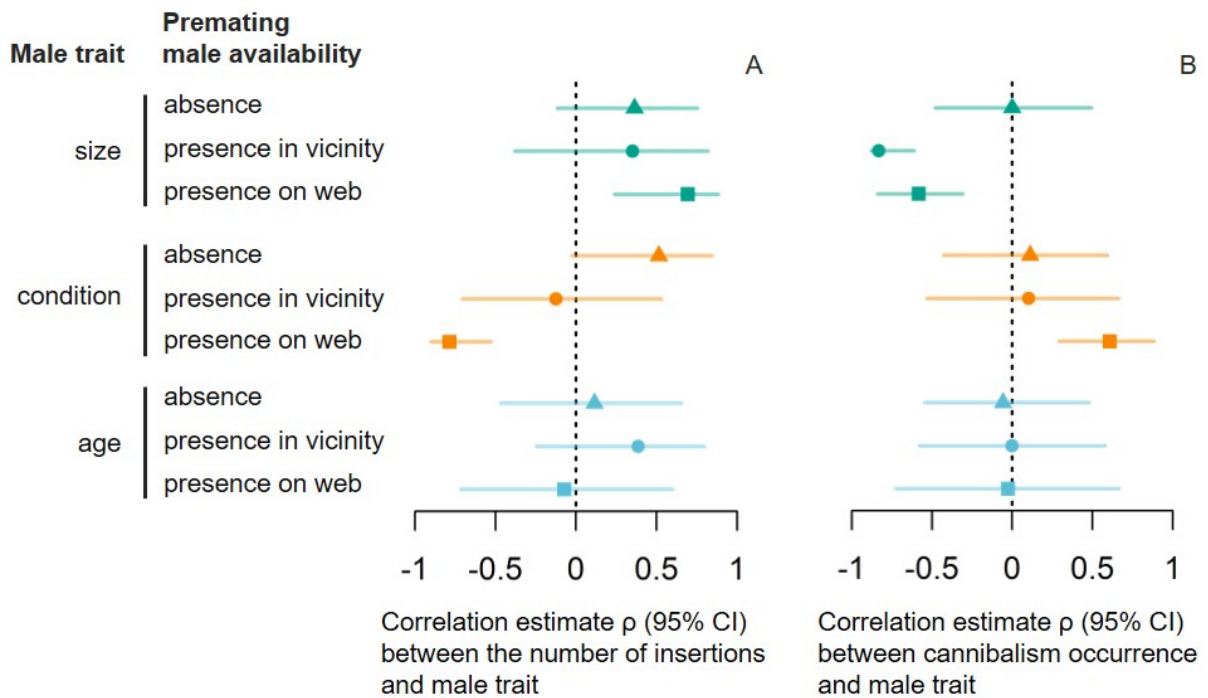


Figure 2:



Supplementary materials S1:

Figure S1: Distribution of standardized size (A, B), condition (C, D) and age (E, F) of males (A, C, E) and females (B, D, F) used in mating trials across pre mating male availability treatments females were exposed to: male absence, male presence in vicinity and male presence on female's web.

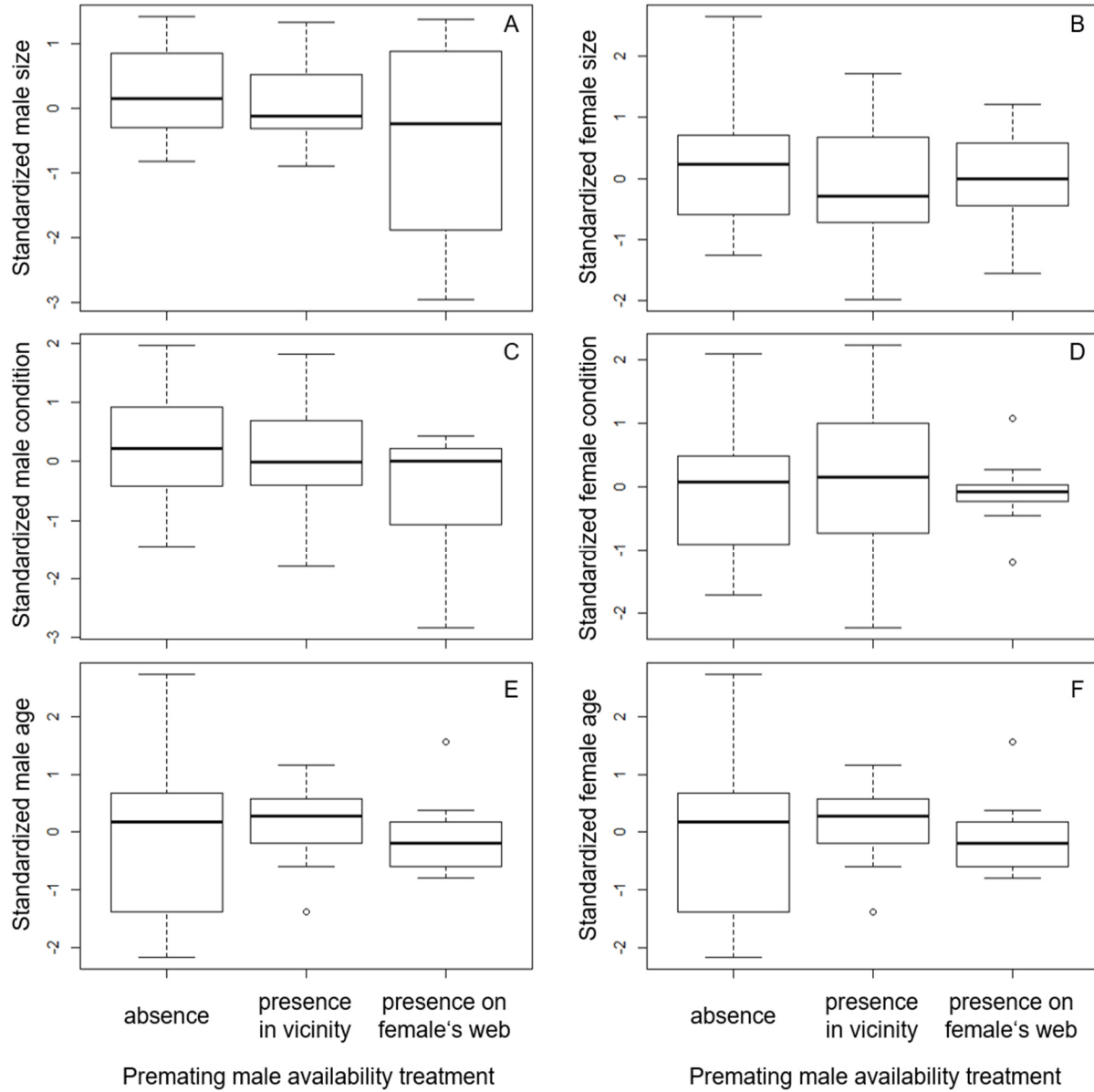


Table S1: Coefficients ρ (95% confidence interval) of the Spearman rank's correlations between the number of insertions or cannibalism occurrence and the standardized male size, condition and age for each pre mating male availability treatment females were exposed to: male absence, male presence in vicinity and male presence on female's web.

Trait	Premating male availability	Number of insertions		Cannibalism occurrence	
		N	ρ (95% CI)	N	ρ (95% CI)
size	absence	17	0.462 (- 0.021; 0.834)	18	0 (- 0.480; 0.494)
	presence in vicinity	14	0.275 (- 0.538; 0.858)	14	- 0.832 (- 0.880; - 0.612)
	presence on web	12	0.787 (0.527; 0.900)	12	- 0.583 (- 0.840; - 0.307)
condition	absence	15	0.515 (- 0.037; 0.837)	16	0.112 (- 0.427; 0.594)
	presence in vicinity	12	- 0.125 (- 0.702; 0.530)	12	0.102 (- 0.530; 0.662)
	presence on web	10	- 0.787 (- 0.901; - 0.527)	10	0.609 (0.292; 0.883)
age	absence	17	0.081 (- 0.535; 0.660)	18	- 0.057 (- 0.547; 0.480)
	presence in vicinity	15	0.327 (- 0.344; 0.783)	15	0 (- 0.579; 0.580)
	presence on web	14	- 0.110 (- 0.894; 0.730)	14	- 0.026 (- 0.727; 0.664)

N refers to the sample size in each group used for the correlation coefficient and 95% CI calculations. *c.f.* Figure 2 or graphical representation.

4. Eigenständigkeitserklärung

Hiermit erkläre ich, dass diese Arbeit bisher von mir weder an der Mathematisch-Naturwissenschaftlichen Fakultät der Universität Greifswald noch einer anderen wissenschaftlichen Einrichtung zum Zwecke der Promotion eingereicht wurde.

Ferner erkläre ich, dass ich diese Arbeit selbstständig verfasst und keine anderen als die darin angegebenen Hilfsmittel und Hilfen benutzt und keine Textabschnitte eines Dritten ohne Kennzeichnung übernommen habe.

I hereby declare that I have submitted this work so far neither at the Faculty of Science and Mathematics at the Greifswald University nor at any other university with the purpose to earn a PhD degree.

Furthermore, I declare that I have written this work as an independent effort and did not use any other sources and guides than those cited in the work. I did not copy any paragraphs of a third author without marking them as a citation.

Greifswald,

Student: Pierick Mougnot

5. Muster der Erklärung zur Abgabe einer elektronischen Kopie der Dissertation

Mathematisch-Naturwissenschaftliche Fakultät

Einverständniserklärung nach § 4 Abs. 1 Nr. c Promotionsordnung

Hiermit erkläre ich, dass von der Arbeit eine elektronische Kopie gefertigt und gespeichert werden darf, um unter Beachtung der datenschutzrechtlichen Vorschriften eine elektronische Überprüfung der Einhaltung der wissenschaftlichen Standards zu ermöglichen.

Datum

Unterschrift

6. Other scientific contributions

Talks:

Mouginot P, Prügel J, Thom U, Steinhoff POM, Kupryjanowicz J, and Uhl G (2016). Securing paternity by mutilating female genitalia. Talk at the 8th Symposium of the Zoological Institutes & Museums, Greifswald (Germany).

Mouginot P and Uhl G (2015). External Female Genital Mutilation, a novel strategy to secure paternity. Talk at the 10th meeting of the Ethological Society, Hamburg (Germany).

Posters:

Mouginot P and Uhl G (2018). The evolution of female genital mutilation: implications of fitness costs and female mate preference. Poster presentation at the 111th meeting of the German Zoological Society (DZG), Greifswald (Germany); and at the 2nd joint congress on evolutionary biology Evolution 2018, Montpellier (France).

Mouginot P and Uhl G (2017). Securing paternity by mutilating female genitalia in an orb-weaving spider. Poster presentation at the ASAB Winter Meeting, Association for the Study of Animal Behaviour, London (United Kingdom).

Mouginot P, Steinhoff POM and Uhl G (2016). Securing paternity by mutilating female genitalia in an orb-weaving spider. Poster presentation at the 7th Conference of the Arachnology Society, Arages, Greifswald (Germany).

Mouginot P, Kupryjanowicz J, and Uhl G (2014). Can males circumvent female mate choice in the orb-weaving spider *Larinia jeskovi*? Poster presentation at the 44th colloquium of the French Society for the Study of Animal Behaviour (SFECA), Paris (France).

Co-authorship of article in preparation:

Fischer K, Kreyling J, Beaulieu M, Beil I, Bog M, Bonte D, Holm S, Knoblauch S, Koch D, Muffler L, **Mouginot P**, Paulinich M, Scheepens JF, Schiemann R, Schmeddes J, Uhl G, van der Maaten-Theunissen M, Weier JM, Wilmking M, Weigel R & P Gienapp (*in prep*). Environmental stress does not increase genetic variance across a diverse group of taxa.

Junghanns A, **Mouginot P**, Lubin Y and Uhl G (*in prep*). Male scarcity and its implications for the reproductive skew in a social spider.

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