A non-sperm transferring genital trait under sexual selection: an experimental approach

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Male genitalia are among the fastest evolving morphological characters, and at a general level sexual selection seems to be involved. But experimental determination of the functions of many remarkable genitalic elaborations is very rare. Here we present the first study to address experimentally the adaptive function of a male genital structure that is not involved in sperm transfer. Females of the orb-weaving spider *Argiope bruennichi* are sexually cannibalistic and polyandrous. The male increases his paternity by obstructing the female's insemination duct with a fragment of his complex genitalia (embolus tip). We manipulated males by detaching another species-specific structure, the median apophysis spur, and found that the spur promotes breakage of the embolus tip inside the female duct, but does not affect the probability and duration of copulation. These data are novel in that they suggest that a genitalic structure which does not transfer sperm nevertheless evolved in the context of sperm competition.

Keywords: sexual selection; genital damage; evolution of genitalia; sperm competition; manipulation of genitalia

1. INTRODUCTION

Male genitalia are among the most complex morphological traits in organisms with internal fertilization and are, therefore, especially useful in distinguishing closely related species throughout the animal kingdom (see Eberhard 1985 for review). Male genitalia may be equipped with species-specific, often bizarre structures, such as spikes, spines and spurs that often play no direct role in sperm transfer (e.g. Cordoba-Aguilar 1999; Crudginton & Siva-Jothy 2000; Cordoba-Aguilar *et al.* 2003; Morrow & Arnqvist 2003). Why these structures exist has puzzled biologists for decades (Eberhard 1985; Hosken & Stockley 2004) and to date, comparative and morphological evidence supports sexual selection as the driving force responsible for their evolution (e.g. Eberhard 1996; Arnqvist 1997; Arnqvist & Danielsson 1999; Danielsson & Askenmo 1999; Tadler 1999; House & Simmons 2003; Wenninger & Averill 2006). The specific mechanisms involved, however, are hotly and controversially debated (Arnqvist & Rowe 2002; Eberhard 2004a,b; Eberhard & Ramirez 2004). An important approach for investigating evolutionary mechanisms is to study a trait's present function by means of experimental manipulation. Such an approach is overdue, and also challenging due to the difficulty in manipulating male genitalia without preventing successful copulation (Arnqvist & Rowe 2005).

Females of the orb-weaving spider *Argiope bruennichi* are sexually cannibalistic and polyandrous while males are monogamous. Males have paired genitalia, the pedipalps, that consist of several sclerites apart from the sperm-transferring structure, the embolus. When males with no

prior mating experience (virgin males) survive their first mating, they usually mate with the same female again (S. H. Nessler, G. Uhl and J. M. Schneider 2006, personal observation). The operational sex ratio is male-biased early in the season such that most males encounter a high risk of sperm competition. Under sperm competition, male relative paternity success is positively correlated with relative copulation duration (Schneider *et al.* 2006). Nessler *et al.* (2007) showed experimentally that males can significantly reduce the copulation duration of rivals by mutilating their emboli to obstruct the insemination ducts of the female with the broken-off fragments (figure 1a–e). Males can expect to father the majority, if not all, of the offspring of that female if they are able to position a fragment inside each of the female's paired insemination ducts (Nessler *et al.* 2007). While males benefit from monopolizing a female through genital damage, females are polyandrous and have not been observed to reject a courting male. Sexual cannibalism after the male's first copulation reduces the male maximal mating rate by half while it increases the female's chance for polyandry (Schneider *et al.* 2006), resulting in a high potential for sexual conflict over mating rates.

In *A. bruennichi*, male genitalia not only consist of the sperm-transferring part—the embolus—that is mutilated during copulation (Nessler *et al.* 2007) but also of several other structures, like the median apophysis (MA), which possess a thin and pointed spur (figure 1b). The presence of a spur is characteristic for the genus *Argiope*, but its shape varies between species (Levi 1983).

In order to gain insights into the selective forces responsible for shaping a genital structure that is not involved in sperm transfer, we performed an experiment in which we detached the spur on the MA (figure 1c). We compared the reproductive success of manipulated and

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Figure 1. Male and female genitalia of *A. bruennichi*. (a) Male (ventral view) with paired genitalia, the pedipalps (arrows). (b) Right pedipalp (unused) with spur (arrow) on MA and embolus tip (circle). (c) Manipulated right pedipalp (used) without spur. Note that the embolus tip is missing. (d) Macerated epigyne (dorsal-lateral view) with stuck embolus tip. (e) Close-up of stuck embolus tip (circle). (f) Female (ventral view) with scapus (arrow). (g) In-copula fixation. Parts of the pedipalp are attached to the scapus (arrow). MA, median apophysis with spur; Ra, radix. Scale bar, 500 μm .

unmanipulated males in terms of the occurrence of (i) genital damage, (ii) stuck genital fragments, (iii) cannibalism, and (iv) the duration of copulation.

2. MATERIAL AND METHODS

(a) *Study animal*

In July 2005, we collected subadult individuals of the araneid spider *A. bruennichi* (Scopoli 1772) from their webs on meadows near Ludwigslust, Germany. In the laboratory, the animals were housed in 330 ml plastic cups and were fed twice a week on a diet of *Drosophila* and watered once per day, 6 days per week. After the final moult, females were transferred to Perspex frames (36 $\times$ 36 $\times$ 6 cm) where they built their typical orb webs. Adult males remained in plastic cups until the start of the mating experiment. Age is given in days from moult to maturity.

(b) *Experimental set-up and mating trials*

Females and males were randomly assigned to three different mating treatments: (i) females were mated to males with unmanipulated pedipalps (S+; $n=23$), (ii) females were

mated to males with the spur of both the left and right pedipalp removed (S-; $n=22$), and (iii) as a control (C), a third group of 10 females was mated to males that had either the spur of the left (C; $n=5$) or the right (C; $n=5$) pedipalp removed while the other pedipalp was left intact. This experimental group helped to assess whether males discriminated between their unmanipulated and manipulated pedipalps and whether copulation duration and occurrence of sexual cannibalism depended on which side they used.

Matings were staged by placing a male in an upper corner of the female's web. Females generally began to swing in the hub and males almost immediately approached the female and commenced courtship. Courtship was always followed by a copulation that always ended with the females attacking the male with or without the result of cannibalism (73% cannibalized; $N_{\text{cannibalized}}=40$, $N_{\text{escaped}}=15$). Since males were removed from the female for morphological investigations, cannibalism is here defined as the capturing without the consumption of the male.

We recorded which pedipalp was used, whether the embolus tip broke off, and if so, whether the broken-off

Table 1. Comparison of mating latency (s), copulation duration (s), female and male age (from final moult to experiment in days), weight (mg) and size (mm) between (A) all copulations with unmanipulated (S+: spur present) and manipulated (S–: spur removed) males and (B) S– males that were successful and unsuccessful in plugging a female (their embolus tip was found stuck in the female insemination duct or not). (Data are given as mean $\pm$ s.e. (N).)

	A				B			
	S+ total	S– total	U-test		S– plug	S– no plug	U-test	
			Z	p			Z	p
mating latency	256.81 $\pm$ 32.53 (26)	210.38 $\pm$ 23.51 (24)	–0.91	0.36	64.00 $\pm$ 20.96 (13)	103.29 $\pm$ 51.03 (7)	0.28	0.78
copulation duration	5.52 $\pm$ 0.75 (28)	5.69 $\pm$ 0.68 (27)	0.52	0.60	6.50 $\pm$ 1.14 (14)	5.82 $\pm$ 0.69 (9)	0.06	0.95
female age	4.00 $\pm$ 0.27 (27)	3.74 $\pm$ 0.32 (27)	0.49	0.63	4.29 $\pm$ 0.41 (14)	3.67 $\pm$ 0.55 (9)	–0.94	0.35
female weight	74.08 $\pm$ 4.8 (28)	74.08 $\pm$ 4.80 (28)	0.51	0.61	78.01 $\pm$ 7.94 (14)	75.97 $\pm$ 5.35 (9)	0.22	0.83
female size	4.98 $\pm$ 0.09 (27)	5.20 $\pm$ 0.10 (26)	1.59	0.11	5.19 $\pm$ 0.15 (14)	5.23 $\pm$ 0.13 (9)	0.13	0.90
male age	4.46 $\pm$ 0.22 (28)	4.26 $\pm$ 0.25 (27)	–0.47	0.64	4.29 $\pm$ 0.30 (14)	4.11 $\pm$ 0.54 (9)	0.00	1.00
male weight	9.30 $\pm$ 0.46 (27)	8.83 $\pm$ 0.57 (27)	–1.30	0.19	9.5 $\pm$ 0.79 (14)	8.28 $\pm$ 1.13 (9)	–1.04	0.30
male size	3.56 $\pm$ 0.09 (19)	3.58 $\pm$ 0.10 (18)	0.15	0.88	3.74 $\pm$ 0.09 (9)	3.44 $\pm$ 0.23 (6)	–1.42	0.16

fragment remained stuck in the female's insemination duct (see below; figure 1d,e). Additionally, we recorded the occurrence of cannibalism and the duration of copulation. Copulation duration was defined as the time between insertion and removal of the pedipalp from the female genitalia. Females were weighed after copulation and males either on the day before or on the same day of the first mating trial with an electronic balance (accuracy 0.01 mg). Male and female body size was measured as the length of tibia plus patella of the first leg by using a microscope and the software LEICA IM v. 4.0.

Six mated females of group S– and of group S+ were transferred into 330 ml cups after the mating trial to allow them to produce an egg sac. Females generally lay eggs within four weeks after copulation. The egg sacs were stored in individual plastic containers at room temperature. After an average of 28 days, we preserved the egg sacs in ethanol and counted the hatchlings and undeveloped eggs under the microscope in order to determine relative and absolute hatching success. Relative hatching success is defined as the proportion of hatchlings divided by the total number of undeveloped eggs and hatchlings; absolute fertilization success is the total number of hatchlings.

(c) Spur manipulation and morphological investigations

We removed the spurs with tweezers while males were anesthetized with CO₂ and additionally chilled on ice. After manipulation, males were given at least 10 min convalescence. We could not observe any impact of spur detachment and sedation procedure on male mating behaviour. All males achieved copulations. Males of the control group were equally likely to use their manipulated or unmanipulated pedipalp (G -test = 1.00, $N_{\text{manipulated}} = 5$, $N_{\text{unmanipulated}} = 5$, $p = 1.00$) for copulation. There was no difference in copulation duration (Mann–Whitney U -test: $Z = 0.63$, $p = 0.53$) or probability of cannibalism (G -test = 2.50, $N_{\text{manipulated}} = 3$ of 5, $N_{\text{unmanipulated}} = 5$ of 5, $p = 1.00$) between males that used the manipulated or unmanipulated side. Moreover, males that were not chilled on ice nor anesthetized (S+ males) did not differ from males of the control group (C) and S– males in the probability of cannibalism (G -test_{3,55} = 2.81, $p = 0.24$)

and copulation duration (ANOVA on Box–Cox transformed copulation duration: $F_{2,55} = 0.004$, $r^2 = 0.0002$, $p = 1.00$). Hence, we added control males that used an unmanipulated pedipalp to the treatment group S+ and control males that used a pedipalp with the spur removed to treatment group S–. We therefore consider groups S+ and S– with a slightly larger sample size in further statistical analysis (S+: $n = 28$; S–: $n = 27$). The two treatment groups did not differ in male and female size, weight and age (table 1).

For morphological investigations, males and females were killed by hypothermia immediately after the end of the trial and were fixed in 70% ethanol. We removed the used pedipalps from the males and inspected them for damage (figure 1c) under the stereomicroscope.

The female's genital region (the epigyne) of individuals that were killed immediately after the mating trials was dissected and macerated with NaOH until it became translucent (figure 1d,f). We determined the plugging success of males by inspecting the used insemination duct for stuck pedipalp fragments (figure 1d,e). Females that were kept to lay egg sacs were killed after an average of 36.09 ± 0.78 ($n = 11$) days after mating trials, but were otherwise treated as described above. The probability of the plug remaining in the insemination duct was not influenced by this time interval (G -test = 2.09, $N_{\text{killed after egg-sac}} = 11$ (7 out of 11 fragments in place), $N_{\text{killed immediately}} = 37$ (31 out of 37 fragments in place), $p = 0.15$).

(d) Data analyses

Data analyses were carried out with JMP v. 4.0.2. All statistical tests are two-tailed ($\alpha = 0.05$). Sample sizes may differ between analyses because not all data were available for each mating trial. Descriptive statistics are given as mean $\pm$ s.e.

3. RESULTS

(a) Effect of genital manipulation on genital damage and plugging success

The probability of genital damage ($N_{S+} = 25/28$, $N_{S-} = 24/27$, G -test = 0.002, $p = 0.96$; figure 2) and cannibalism ($N_{S+} = 19/28$, $N_{S-} = 21/27$, G -test = 0.68, $p = 0.41$; figure 2) did not differ between males that used a pedipalp

Figure 2. Comparison of the frequency of cannibalism, genital damage (broken-off embolus tips), plugging (stuck embolus tips) and fertilization success between males with untreated (S+; white bar) and manipulated (S-; black bar) pedipalp. n.s., not significant. *** $p \leq 0.001$.

with (S+) or without (S-) a spur. However, males that used a pedipalp without a spur (S-) were less successful in placing the embolus tip inside the female's insemination duct (14/23) than males that used a pedipalp with spur (S+; 24/24, G -test = 11.62, $p = 0.0007$; figure 2).

S- males that successfully positioned a plug did not differ in age, weight, size, copulation duration or the frequency of cannibalism (G -test_{2,23} = 0.002, $p = 0.96$) from S- males that could not position a mating plug. Females of these two groups did not differ in age, weight and size (table 1).

(b) Effect of genital manipulation on fertilization success

We could not find a difference in relative (U -test: $Z = 0.08$, $N_{S+} = 6$, $N_{S-} = 6$, $p = 0.94$; figure 2) or absolute hatching success ($N_{S+} = 64.67 \pm 15.94$ developed eggs, median = 62.5; $N_{S-} = 66.5 \pm 13.58$ developed eggs, median = 77.5; U -test: $Z = 0.24$, $N_{S+} = 6$, $N_{S-} = 6$, $p = 0.81$) between females mated to S- or S+ males.

4. DISCUSSION

Removal of the spur had significant consequences for the manipulated males in that they were less likely to position the tip of the embolus inside the insemination duct of the female and thereby were less successful at preventing sperm competition. Since sperm competition is a prominent feature of the mating system of *A. bruennichi* (e.g. Fromhage & Schneider 2005; Schneider *et al.* 2006; Nessler *et al.* 2007), we suggest that the spur is an adaptive male trait that evolved under sexual selection.

Careful observations of the copulations revealed no differences in mating behaviour, exemplified by no significant differences in copulation duration and sexual cannibalism, between manipulated and untreated males; nor was there any apparent difference in females that mated with males of either group. Sham control males that had one intact and one manipulated pedipalp did not preferentially use their intact pedipalp nor did they behave differently from unmanipulated males. This further increases our confidence that the handling had no effect on male copulation behaviour.

In earlier studies with unmanipulated males, significant positive relationships were established between the duration of copulation, the amount of sperm transferred and paternity success under sperm competition (Schneider *et al.* 2006). The facts that spiderlings hatched from all egg sacs and that hatching success was not different between

treatment groups suggest that sperm transfer and fertilization were not affected by the manipulation. Although sample sizes were relatively small, very similar means and standard deviations suggest that a larger sample size would not have changed the result.

We dissected most females directly after copulation in order to prevent an underestimation of plugging success due to embolic tips that had fallen out. Although this has been observed in other spiders of the genus *Argiope* (S. H. Nessler 2006, personal observation), we now know that the precaution was unnecessary in our system.

Males lacking the spur differed in only one of the observed components of the mating behaviour from males with untreated pedipalps: they were on average less successful in leaving the small tip of the sperm-transferring embolus inside the female insemination duct. A previous study demonstrated that the embolus tip obstructs the insemination duct and thereby reduces fertilization success of future rivals (Nessler *et al.* 2007). Genital damage by males of *Argiope bruennichi* thus may have evolved as a means to prevent or reduce sperm competition. Similar adaptations have been shown in other spider species (Fromhage & Schneider 2006; Snow *et al.* 2006). *A. bruennichi* males use each of their pedipalps only once, and most pedipalps are visibly damaged after use. Whether loss of the tip causes sterility is unknown for our system, but in other spider species a causal connection was dismissed (Breene & Sweet 1985; Snow *et al.* 2006). Nevertheless, mating rates of male *A. bruennichi* are very low due to sexual cannibalism: only approximately 20% of all males survive their first insertion (Fromhage *et al.* 2003; Schneider *et al.* 2006). Surviving males have two options: they can either return and empty their second pedipalp in the previously unused side of the same female or search for another mating partner. Since females are polyandrous, males benefit from protecting their sperm by leaving a mating plug behind. Interestingly, males without a spur managed to break off the tip of the embolus, but only 60% of them were able to correctly place the plug inside the female. These two groups of manipulated males did not significantly differ in copulation duration, age, weight and size or probability of cannibalism nor between the females they mated with. In-depth morphological studies are underway, which will clarify the complicated mechanics of genital coupling in these spiders. In-copula fixations (figure 1g) tentatively suggest that the spur helps to guide the sperm-transferring embolus into its optimal position such that its tip accurately fits into the female insemination duct and/or may ensure that the tip remains inside the female when she attacks and starts to dislodge the male (Uhl *et al.* in press).

The present study clearly demonstrates that the spur indirectly reduces sperm competition by promoting the effective use of the embolus tip to plug the insemination duct of the female. However, whether sperm competition alone is responsible for the evolution of the spur remains unclear. In addition to sperm competition, two further scenarios are conceivable: (i) the spur has evolved under sexual conflict by helping males to monopolize females against the latter's best interest (Arnqvist & Rowe 2005) and/or (ii) the spur has evolved under cryptic female choice in that females indirectly benefit from matings with males with high plugging efficiency (Eberhard 1985). To date, we can neither exclude nor support the sexual

conflict or cryptic female choice hypotheses as alternative or additional explanations to sperm competition. However, our study system provides us with a powerful tool that can be used for further experiments. Furthermore, since probability of cannibalism, shape of the spur and female genitalic morphology varies considerably in the genus *Argiope*, a comparative approach may eventually enable us to disentangle the evolutionary forces responsible for shaping genitalia in *A. bruennichi*.

In summary, our study is, to our knowledge, the first experimental support that demonstrates that a genitalic but non-sperm transferring trait has a sexually selected function and thus supports the hypothesis that sexual selection is a powerful force behind the evolution of elaborate genitalia.

The research was supported by a DFG grant to J.M.S. We thank L. Fromhage and two anonymous referees for their helpful comments on the manuscript.

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