Male copulatory sensory stimulation induces female ejection of rival sperm in a damselfly

Alex Córdoba-Aguilar

Department of Animal and Plant Sciences, University of Sheffield, Sheffield S10 2TN, UK (sclab@sheffield.ac.uk)

Male damselflies possess very specialized genitalia. Females mate multiply and store sperm in two sperm storage organs, the bursa copulatrix and the spermatheca. During copulation, males physically remove the sperm stored in these organs using their genitalia. I document a novel mechanism by which males gain access to the spermatheca in *Calopteryx haemorrhoidalis asturica*. The mechanism is based on male stimulation of the female sensory system that controls egg fertilization and laying. During copulation, the aedeagus (a male genitalic structure indirectly involved in sperm transfer) distorts the cuticular plates in the female genital tract that bear mechanoreceptive sensilla. This stimulation results in sperm ejection from the spermatheca. Aedeagus width is positively correlated with the amount of sperm ejected. I propose that males have exploited a pre-existing female sensory bias to gain access to otherwise physically unreachable sperm. These results shed light on the issue of the origin of female preferences in current models of sexual selection and on the evolution of genitalia via sexual selection. It is postulated that females might use this process as a form of post-copulatory sexual selection on the basis of males' genitalia.

Keywords: sexual selection; sensory exploitation; sperm competition; fertilization; damselfly

1. INTRODUCTION

Robust evidence suggests that the evolution of extravagant male characters (e.g. cricket, frog and bird songs, the sword of swordtail fishes, deer antlers) is due to male–male competition, female choice or both processes (Darwin 1871; Andersson 1994). However, many aspects of sexual selection theory still remain to be clarified. This is the case for the origin of female preferences for such characters in males (Ryan 1997).

Traditional sexual selection models stress a coevolution of the male trait and the female preference. A more recent model suggests that female preferences pre-date the male trait (West-Eberhard 1984; Burley 1985; Kirkpatrick 1987; Ryan *et al.* 1990; Basolo 1990, 1995a,b; Kirkpatrick & Ryan 1991; Proctor 1992; Enquist & Arak 1993; Ryan & Rand 1993). In this 'sensory exploitation' model, the preferences are viewed as sensory biases that evolved in a different context. Sexual selection via sensory exploitation postulates that males with traits matching the pre-existing female 'preference' were favoured by females during mate choice. In swordtail fishes, for example, there is good evidence that female preference for the male sword evolved before the appearance of the male trait (Basolo 1990, 1995a). It has been suggested that this sensory exploitation scenario can occur in other species (Ryan & Rand 1993) and evidence from several taxa suggests that this might be so (Basolo 1990; Ryan *et al.* 1990; Proctor 1992; McClintock & Uetz 1996).

Pre-existing biases have been divided into three types (Basolo 1995b): (i) an adaptive bias that is used to satisfy needs not related to sexual selection; (ii) a currently

neutral bias which had an adaptive value in the past; and (iii) a bias that never has had a function but has not been selected against. Although evidence supporting the sensory exploitation hypothesis is accumulating, very little is known about the nature of the female bias (however, see Proctor 1991, 1992).

A modified version of the sensory exploitation hypothesis has been proposed to explain patterns of genitalic evolution and diversity (Eberhard 1993). According to this version, 'arbitrary' genitalic stimuli might have evolved exploiting female sensory biases during copulation. As a product of changes in the female preferences and some males being more able to produce more intense stimuli, such exploitation would result in a runaway process (Fisher 1930). Although the patterns of genitalic diversity can alternatively be seen as a product of conflict between sexes over reproduction (rather than a female choice process; Arnqvist & Rowe 1995; Rice 1996; Alexander *et al.* 1997), it is clear that more detail of the mechanisms underlying reproductive processes during and after copulation is needed to explain genitalic evolution.

Odonate insects (dragonflies and damselflies) have been extensively used in studies of sperm competition (the competition among different males' sperm present in the female's genital tract for ovum fertilization (Parker 1970)). As a consequence, sperm precedence mechanisms are very well understood in different species (Waage 1979; Miller 1987; Siva-Jothy 1988; Siva-Jothy & Tsubaki 1994; Cordero & Miller 1992). In calopterygid damselflies, for instance, two distinct behavioural stages have been identified: sperm removal followed by sperm transfer (Waage 1979). Sperm are stored by the female in specialized

Figure 1. (a) Vaginal plates showing campaniform sensilla, magnification is $10\times$. (b) Aedeagus showing the width measurement used. Scale represents 0.5 mm.

organs, the bursa copulatrix and the t-shaped spermatheca. In males of the damselfly *Calopteryx maculata*, the penis head and the projections on it appear, each structure, to remove sperm differentially from both sperm storage organs. The penis head removes bursal sperm and the two distal projections of the penis do the same for the sperm stored in the spermatheca (Waage 1979).

Here, I examine the mechanism responsible for changes in the volume of sperm stored within the female sperm storage organs in the territorial damselfly *Calopteryx haemorrhoidalis asturica*. Despite the morphological similarities in genitalia with *C. maculata*, the mechanism underlying the change in volume of spermathecal sperm in *C. h. asturica* is not physical. The mechanism is related to the female sensory system involved in controlling egg fertilization and laying.

The female genital tract in odonates has two lateral sclerotized cuticular plates, each bearing campaniform sensilla (figure 1a) which coordinate sperm ejection during egg laying (Miller 1987; Siva-Jothy 1987). Fertilization stimuli are produced by the egg forcing the cuticular plates apart and thereby distorting the cuticle. Stimuli go to the eighth abdominal segment ganglion, and then to

the sperm storage organs (Miller 1987). As a response, stored sperm are ejected from the sperm storage organs from a special pore where fertilization takes place (Siva-Jothy 1987).

During copulation, the male intromittent organ must also stimulate the female sensory system and produce the same egg effect so that females eject sperm. In this study I examine whether males have exploited the female sensory system and thereby gain access to other males' stored sperm. My findings provide evidence in support of the sensory exploitation hypothesis as well as that on the evolution of animal genitalia via sexual selection. Especially relevant for the former paradigm, this study sheds light on the nature and function of the female bias.

2. METHODS

I studied a population of *C. h. asturica* in Lourizán, near Pontevedra, Spain in the summers of 1996, 1997 and 1998. To measure the change in the volume of sperm stored in the female sperm storage organs (and in the experimental protocol) I interrupted hand-paired (Oppenheimer & Waage 1987) animals at specific behavioural events during copulation (see Siva-Jothy & Hooper 1995). All animals were preserved in 70% ethanol and rehydrated 24 h before dissection (except for observations of genitalic coupling, see below). I used the method of measuring and comparing the external dimension of sperm masses to assess the degree of sperm displacement. This method has proved to be highly reliable in zygopterans to give a relative and constant measure of sperm density. This is because the zygopteran sperm masses are dense and compact (Miller 1984; Waage 1984; A. Córdoba-Aguilar, personal observations) and are not volumetrically affected by different preserving substances (Waage 1979, 1984). Sperm masses were placed on a slide preparation beneath a coverslip. The mass was then squashed to a known, constant thickness (having the coverslip held up by two coverslips on either sides) and their outlines drawn to scale on a piece of paper using a *camera lucida*. I then calculated the sperm volume (in mm^3) using the weight of the cut-out sperm drawings using the appropriate scale constants.

Preliminary results indicated that the volumes of spermathecal sperm changed during copulation. I therefore examined whether the appendages on the penis head (figure 2a) had physical access to the spermatheca, as occurs in *C. maculata* (Waage 1979). I investigated this using three approaches. The first was based on genitalic measurements. I measured and compared the following traits: the width of each flat lateral penis horn (figure 2a) and the width (i.e. half circumference) of each flattened spermathecal duct (figure 2b). The middle point that divided the total length of each structure in two similar distances was used as a reference point to measure the width of that structure. However, as the distal parts of the penis horns are narrower than their central regions, I also measured and compared the diameter of the circumference produced at the curved tip of the horns with the width (half circumference) of the flattened spermathecal ducts at their basal region.

The second approach was based on direct observation of genitalic coupling. I interrupted copulating pairs at times where sperm displacement was still occurring (between the 20th and 50th abdominal flexions, see figure 3). This procedure has been used in several studies to document the female genitalic places to which the male genitalia have access (Waage 1979; Cordero & Miller 1992). I approached hand-paired copulating pairs and

Figure 2. Genitalia of both sexes. (a) Male. Distal projections of the penis head. (b) Female. Spermathecal ducts. Scale represents 0.5 mm.

killed them instantly by trapping them between my clapped hands (holding the pair to avoid them falling to the ground). This prevented the animals from separating. Immediately after, I cut off the abdomens and preserved them for future observations of the still-engaged genitalia under a dissecting microscope.

A third source was used to investigate if physical removal of spermathecal sperm was occurring. I documented the distribution of the sperm masses remaining in the spermatheca in those females interrupted at their 50th abdominal flexion (the point during copula where sperm storage organs hold least sperm, see figure 3). The assumption was: if physical removal is occurring, the more likely places where sperm masses (if any) will remain after removal should be in the tips of the spermathecal ducts

Figure 3. Volumes (means $\pm$ s.e. bars) of sperm in the bursa and the spermatheca of non-virgin females from different events: pre-copula (pre-cop), during (divided according to the number of abdominal flexions that the pair characteristically carries out while in copulation) and post-copula (post-cop). Numbers in parentheses are sample sizes for both organs.

(as those would be the furthest places and, therefore, last to be emptied) as happens, for example, in *C. maculata* (Waage 1979).

My results suggested a different mechanism to explain the change in spermathecal sperm volume during copulation. Miller (1987, 1990) suggested that copulatory movements of the male aedeagus shaft against the vaginal plates that bear the campaniform sensilla could stimulate sperm release from the spermatheca. To explore this hypothesis, I investigated if there was a relationship between the female sensory system that controls egg laying and the amount of sperm released. Thirty-five copulating non-virgin females were interrupted immediately after the 50th abdominal flexion. I measured the number of campaniform sensilla on each plate and the amount of sperm present in each spermathecal duct.

My results led me to examine further if the size of the male's genitalia had a role on the volume of sperm ejected. The shaft of the male's aedeagus (figure 1b) is the only structure that is in contact with the vaginal plates during copulation. I reasoned that the wider the aedeagus, the more deformations of the plates it will produce. As sensilla respond to deformations of the surface where they are imbedded, more deformations will result in more sperm being released. This was tested by simulating in-copula movements of the male's genitalia using dissected aedeagi. These structures had their penis heads (the entire structure shown in figure 2a) removed (so physical removal could not occur) and differed only in the width of the part that is in physical contact with the vaginal plates during copulation (figure 1b). Each aedeagus was used with five different non-virgin, live females. Each manipulated copulation consisted of 50 in-and-out movements of the aedeagus produced at similar speed and motion to a natural mating (over 100–103 s). I preserved the females immediately after the treatment.

Male genitalic structures were permanently mounted in pyccolite on glass slides. Female genitalia were preserved in 70% ethanol. Morphometric measurements (in millimetres) were made using Optimas[®] image analysis software via a video camera attached to a compound microscope. Means are shown $\pm$ s.d.

3. RESULTS

(a) *Changes in the volumes of sperm stored by females during copulation*

There was a reduction in the volume of sperm in both sperm storage organs during the first stage of copulation (figure 3). The lowest volume of sperm in both organs was found at the 50th abdominal flexion during the first stage of copula. After this behavioural event, the sperm increased in both organs. I found sperm masses attached to the penis head and distal projections during this stage. This suggests that sperm stored in the bursa are physically removed during copulation, as in other calopterygid damselflies (Waage 1979, 1988; Siva-Jothy & Tsubaki 1989; Siva-Jothy & Hooper 1995).

(b) *Possible mechanisms underlying the change in spermathecal sperm volume during copulation*

(i) *Physical removal*

The genitalia of 20 males and females were measured and compared. In every comparison the male genitalia was wider than that of the female: for the middle regions of the penis processes and the spermathecal ducts (right penis process versus right spermathecal duct, 0.45 ± 0.02 and 0.36 ± 0.03 , respectively, Student's t -test = 11.4, d.f. = 35, $p < 0.0001$; left penis process versus left spermathecal duct, 0.44 ± 0.02 and 0.36 ± 0.03 , respectively, Student's t -test = 9.4, d.f. = 36, $p < 0.0001$), and for the tips of the penis processes and the basal parts of the spermathecal ducts (right penis process versus right spermathecal duct, 0.072 ± 0.016 and 0.056 ± 0.009 , respectively, Student's t -test = 3.84, d.f. = 30, $p = 0.0006$; left penis process versus left spermathecal duct, 0.074 ± 0.018 and 0.058 ± 0.008 , respectively, Student's t -test = 3.61, d.f. = 26, $p = 0.001$).

To make direct observations on genitalic coupling, 25 copulating pairs were interrupted. In all cases the penis horns and head were found in the bursa copulatrix. Consistent with the result of genitalic measurements, no male genitalic structure was found in the spermathecal ducts.

Finally, of those females interrupted at their 50th abdominal flexion the sperm masses remaining in the spermathecae were not distally orientated but were patchily distributed in both ducts (see, for example, figure 2b).

These three sources of evidence, as well as the inelastic nature of the sperm storage organs (which tear when gently stretched by forceps due to their cuticular and chitinous histological constitution: Davey 1985; Srivastava & Srivastava 1992; Andrew & Tembhare 1996; A Córdoba-Aguilar, unpublished data), suggest that physical removal of spermathecal sperm by the male genital processes is unlikely. The lateral projections of the male intromittent organ are too wide.

(ii) *Removal via sensory stimulation*

Of the 35 females interrupted at their 50th abdominal flexion, most had an asymmetry in the number of sensilla present in each of the vaginal plates (20 had more sensilla on the left plate, six on the right and nine were symmetrical, although the total numbers of sensilla in each group were similar (means $\pm$ s.d.; left-biased = 54.3 ± 8.3 , right-biased = 53 ± 8 , symmetrical = 49.4 ± 5.6 ; Kruskal-Wallis test = 2.13, $p = 0.34$). The magnitude of the asym-

Table 1. Relationship between the proportion of sperm remaining in each spermathecal duct and the number of campaniform sensilla on left and right vaginal plates in non-virgin females interrupted immediately after the 50th copulatory abdominal flexion

(Fisher exact test, $p = 0.0001$. Numbers in parentheses are females interrupted before copulation. Same test, $p = 0.38$.)

	proportion of sensillum on each genital plate	
	left-biased	right-biased
females with biased sperm storage		
left side with less sperm	18 (6)	0 (2)
right side with less sperm	2 (6)	6 (3)

Figure 4. Relationship of aedeagus width with the sperm remaining in both spermathecal sides after the experimental treatment.

metry in sensilla number in both plates was high (number of females differing from one to two sensilla = 10, from three to four = 12, from five to six = 2 and from seven to eight = 2). Using these 26 asymmetrical females (the nine symmetrical females showed no difference in stored sperm volumes between the right (0.035 ± 0.003) and left (0.036 ± 0.002); paired Student's t -test = 0.6, $p = 0.54$), I found an inverse relationship between the number of sensilla in each plate and the amount of sperm present in the spermathecal duct on that side (table 1). This result is not explained by a bias in sperm distribution before copulation (table 1) or by differences in the widths of the medium parts of both spermathecal ducts (means $\pm$ s.d. in mm; width of left duct, 0.035 ± 0.003 , right duct, 0.036 ± 0.003 ; paired Student's t -test = 0.63, $p = 0.54$). These data suggest there is a relationship between the change in spermathecal sperm volume and the distribution of sensilla in the female lateral vaginal plates responsible for egg fertilization.

(c) *Sperm removal via sensory stimulation hypothesis: test and observations*

The experimental treatment manipulating aedeagus size revealed a negative relationship between the amount of sperm stored in each spermathecal duct and the aedeagus width ($y = 0.0672 - 0.103x$, $F_{1,13} = 10.3$, $p = 0.007$;

figure 4). Further analysis between the aedeagus width and the amount of sperm remaining in the spermatheca from those 35 copulating pairs that were interrupted at the 50th abdominal flexion supported this experimental conclusion ($r = -0.57$, $p < 0.01$).

4. DISCUSSION

Several male-driven mechanisms that reduce the risk of sperm competition have been described in insects (reviewed by Simmons & Siva-Jothy 1998). In odonates, mechanisms are manifest as sperm repositioning (Waage 1984; Siva-Jothy 1988) and sperm removal (Waage 1979; Miller 1987). Waage (1979) demonstrated that during copulation, but prior to sperm transfer, *C. maculata* males physically removed the sperm deposited by previous males in the female's sperm storage organs (the bursa and the spermatheca). Males of this and other calopterygid species possess a specialized genitalia consisting of two curved horns and an extensible head. Waage (1979) proposed that the horns removed the sperm present in the spermatheca whilst the head removed the sperm present in the bursa. As genitalic morphology is fairly conserved in the genus *Calopteryx*, several authors (Waage 1984, 1986; Adams & Herman 1991) predicted that the ability to remove rival sperm was widespread in calopterygids.

The genitalic morphology of male and female *C. h. asturica* conforms to the *Calopteryx* pattern and males also displace sperm from both of the female's sperm storage organs. However, the mechanisms involved differ from that in *C. maculata*. The curved horns of the male genitalia mechanically remove sperm from the bursa but do not have access to the spermathecal sperm because they are too large. These differences in dimensions are in agreement with the pattern expected if males were not able to physically and mechanically remove spermathecal sperm because of size differences in both sexes' genitalic structures. In species where physical removal of spermathecal sperm occurs (e.g. *C. maculata*, Waage 1979; *C. virgo*, A. Córdoba-Aguilar, unpublished data), morphometric analysis indicates that horns are narrower than the spermathecal ducts and the reverse occurs for species where males have no access to these female structures (e.g. *C. splendens xanthostoma*, Siva-Jothy & Hooper 1995; A. Córdoba-Aguilar, unpublished data) as the horns are too wide.

Change in spermathecal sperm volume during copulation in *C. h. asturica* must therefore be brought about by a different mechanism. My results suggest that males stimulate the female sensory system that is responsible for the reflex that releases sperm from the spermatheca during fertilization (Miller 1987, 1990; Siva-Jothy 1987). When an egg is oviposited, it passes by, and stimulates mechanoreceptive sensilla embedded in two sclerotized plates at the entrance of the vagina. Such stimulation induces the spermathecal muscles to contract (via impulses from the eighth abdominal segment ganglion) and eject stored sperm. The sperm then pass through a special conduit in the egg which is then fertilized (Miller 1987). During copulation in *C. h. asturica*, the male's aedeagus also stimulates these sensilla and induces the same sperm-releasing phenomenon. To my knowledge, this is the first time that direct evidence has been provided on how this mechanism might work despite findings suggesting similar mechan-

isms in other insects (Miller 1987; Von Helversen & Von Helversen 1991; reviewed by Eberhard 1996). Considering that the anatomical characteristics of the egg fertilization system shown here are similar to those of other insect species, it is possible that this form of sensory exploitation by males might be widespread in insects.

The results from this study also have some heuristic relevance to the hypotheses put forward to explain the evolution of animal genitalia (Eberhard 1985). There are three main hypotheses based on sexual selection theory that attempt to explain genitalic diversity. First, the competition among males for fertilization through their gametes (sperm competition; Parker 1970; Waage 1979). Second, female mechanisms influencing male paternity success (post-copulatory sexual selection; Lloyd 1979; Thornhill 1983; Eberhard 1996). Third, the conflict between sexes for the control of reproduction (Arnqvist & Rowe 1995; Rice 1996; Alexander *et al.* 1997). According to the post-copulatory sexual selection hypothesis, males try to induce females to use their sperm through copulatory courtship (*sensu* Eberhard 1996). Females might use discrete, intraspecifically variable sets of preferences and would choose males according to the copulatory performance of their internal genitalic 'displays'. Although different mechanisms of 'displays', via the use of genitalia, have been proposed (see Eberhard 1996), little documentary evidence has been found. The findings of this study are in accordance with the evolution of genitalia via sexual selection and also provide tentative support for a post-copulatory sexual selection process with a potential male copulatory display. Copulatory stimulation varies between males: those that stimulate females more intensively produce a higher degree of rival sperm ejection. Using a post-copulatory sexual selection scenario in this species, the female's sensory system would play a determining role in this process as it might function as the setting (in terms of number and biases of sensilla) establishing the basis for the preference (wider aedeagi). A paternity analysis is currently under way to examine the fitness consequences of this ability for males.

Current models of sexual selection predict a coevolution of the female preference and the male trait. Of these, the 'sensory exploitation' hypothesis has received substantial attention. This postulates that female preferences pre-date the male traits and that males exploit these pre-existing biases. Convincing evidence has been provided to support this hypothesis (Basolo 1990; Ryan *et al.* 1990; Proctor 1992; McClintock & Uetz 1996). However, how the female preference could have evolved remains a largely unexplored topic. My findings not only provide evidence for the sensory exploitation hypothesis but also elucidate the function of the pre-existing female bias. In *C. h. asturica*, the female sensory system is used for egg fertilization, an essential reproductive function. If the fertilization mechanism (sensilla and sperm storage organs) evolved before the male ability to stimulate it, my results suggest that males have evolved traits that exploit the female's fertilization system to remove inaccessible spermathecal sperm. Moreover, a male's ability to stimulate the female sensory system varies: some males appear to induce higher sperm-releasing responses. The results from this study suggest that the exploited female bias may have two roles: as a coordinator of egg fertilization and as a mate preference setting.

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