# SPERM DISPLACEMENT ABILITY IN
## *CALOPTERYX HAEMORRHOIDALIS* (VANDER LINDEN):
## MALE AND FEMALE ROLES, MALE LIMITS IN PERFORMANCE,
## AND FEMALE NEURAL CONTROL
## (ZYGOPTERA: CALOPTERYGIDAE)

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During copulation, *C. haemorrhoidalis* ♂ ♂ displace the sperm of rivals stored by the ♀. During displacement, sperm stored in 2 spermathecae are ejected by the ♀ as a consequence of ♂ genitalic stimulation: the aedeagus distorts 2 vaginal plates in which mechanoreceptive sensilla are embedded. The sensilla control spermathecal sperm release and a wider aedeagus displaces more sperm. There is variation between ♀ ♀ in their sensillum number which might also affect sperm displacement rate. The role of sensillum number and aedeagal width in sperm displacement variation in copulations whose duration was controlled was investigated. Results indicated that only aedeagal width could predict sperm displacement variation. The neural communication between the vaginal plates and both spermathecae was also examined. Previous observations suggested a "lateral" control of sperm ejection between each vaginal plate and its corresponding spermatheca. This was corroborated by stimulating the mechanoreceptive sensilla of ♀ ♀ that underwent different surgical treatments: only those ♀ ♀ whose vaginal plate nerves were cut, showed no volumetric decline in sperm in the corresponding spermatheca. Finally, the effect of copula duration (number of aedeagal copulatory movements) on sperm ejection was experimentally examined. In natural conditions, ♂ ♂ perform approximately 50 aedeagal movements during copulation. There was no difference in sperm volumes between the pairs of ♀ ♀ that were subjected to 50 and 80 aedeagal movements of stimulation using the same aedeagus. These results help to understand the nature of the spermathecal sperm displacement mechanism in this sp.

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## INTRODUCTION

Sperm competition theory predicts that male traits that increase fertilisation success during and after copulation will be favoured by sexual selection (PARKER, 1970). In agreement with this prediction, a number of mechanisms that either prevent the female from remating immediately and/or increase the chances of the last copulating male's sperm being used for fertilisation have been documented (for a review see SIMMONS & SIVA-JOTHY, 1998). In the Odonata, syn- and post-copulatory mechanisms have been well documented but much more attention has been paid to the former. For example, it is known that males of some species are able to remove, reposition or induce female ejection of rival sperm (reviewed by CÓRDOBA-AGUILAR et al., 2003).

Copulation in damselflies is characterised by a series of abdominal flexions that both sexes carry out (see, for example MILLER & MILLER, 1981). These flexions are related to the internal genitalic processes occurring in the female tract (CÓRDOBA-AGUILAR et al., 2003). Two functional copulatory stages have been identified based on behavioural and physiological aspects of these flexions (MILLER & MILLER, 1981). During stage I, the male genitalia displace sperm present in the sperm storage organs while sperm transfer occurs in stage II.

Males of the territorial damselfly *Calopteryx haemorrhoidalis* displace rival sperm stored in the female's sperm storage organs (the bursa copulatrix and two connected spermathecae) by simultaneously using two mechanisms (CÓRDOBA-AGUILAR, 1999). Most bursal sperm is physically removed by the penis head and its two lateral, distal horns. By contrast, displacement of spermathecal sperm is affected by a mechanism which involves the stimulation of mechanoreceptive sensilla in the female vagina by the male aedeagus (CÓRDOBA-AGUILAR, 1999, 2002). The sensilla are embedded in a pair of sclerotised plates (hereafter vaginal plates) which are located at the entry of the vaginal tract and are used for the control of egg laying and fertilisation (MILLER, 1987; SIVA-JOTHY, 1987a; CÓRDOBA-AGUILAR, 2003). During egg laying, an egg distorts the vaginal plates in which the sensilla are embedded. These sensilla control spermathecal muscle activity through a ganglion present in the VIII abdominal segment, and stimulation by the eggs induce the sperm storage organ muscles to contract and release stored sperm (MILLER, 1987; CÓRDOBA-AGUILAR, 2003). During stage I of copulation, male aedeagal stimulation appears to mimic the presence of an egg and thereby induces the same sperm releasing effect. The volumes of ejected sperm are variable and are directly related to the width of the aedeagus (CÓRDOBA--AGUILAR, 1999). However, females show inter-individual variation in the number of sensilla present in their vaginal plates (CÓRDOBA-AGUILAR, 1999, 2003). The possibility exists that this variation might contribute to variation in the degree of spermathecal sperm displacement alongside the known effect of aedeagus width.

Females also vary intra-individually in the number of sensilla in each vaginal plate: (a) some females have more sensilla in the right vaginal plate; (b) some females have more on the left vaginal plate; and, (c) some females have the same number in both vaginal

plates (CÓRDOBA-AGUILAR, 2003). This pattern and the observations that: (a) the more sensilla a plate has, the more sperm is ejected from the corresponding spermatheca during aedeagal stimulation (CÓRDOBA-AGUILAR, 1999); and, (b) the number of undulating movements by each spermatheca is positively correlated with the number of sensilla in its corresponding vaginal plate after egg-mediated stimulation (CÓRDO-BA-AGUILAR, 2003), suggested a neural communication between a vaginal plate and the corresponding spermatheca.

Abdominal flexions during stage I cause in and out movements of the aedeagus in the female vagina and it is known that the number of such flexions is negatively correlated with the volume of sperm remaining in the spermatheca during this stage (CÓRDO-BA-AGUILAR, 1999) despite the fact that the number of flexions is relatively constant (mean ± STD = 50.2 ± 7.2; CÓRDOBA-AGUILAR, 2000) in natural copulations, there is still considerable variation in spermathecal sperm displacement at the end of copula. This might result from differences in male stimulatory ability (CÓRDOBA-AGUILAR, 1999), but if this is the case it is unclear why males do not extend stage I for longer and thereby produce a higher sperm ejection response.

This work has three objectives. First, we examined the role of inter-individual variation in sensillum number and aedeagal width in affecting spermathecal sperm displacement ability. Second, we experimentally investigated whether there is a neural communication between a vaginal plate and the corresponding spermatheca. Third, because the number of stage I flexions determines the rate of sperm ejection, we investigated whether males gain a displacement advantage by experimentally increasing the number of stage I flexions.

## MATERIAL AND METHODS

GENERAL EXPERIMENTAL PROTOCOL. — A population of *C. haemorrhoidalis* was studied in a stream near Pontevedra, Galicia, Spain in summer 1996, 1997, 1998 and 2002. The experiments detailed here were carried out in this time. To ensure female sperm storage organs were full of sperm before manipulations, females were hand-paired (OPPENHEIMER & WAAGE, 1987). After manipulation, animals were immediately preserved in ethanol (70%) for the subsequent quantification of sperm volume.

The methodology for measuring sperm volumes was as used by SIVA-JOTHY & HOOPER (1995). 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. The sperm volume (in $mm^3$) was then calculated from the weight of the cut-out sperm drawings using the appropriate scale constants. Since it was only spermathecal sperm, but no bursal sperm, what we were interested in, whenever we refer to sperm we mean spermathecal sperm.

Due to the occurrence of zero values and in order to use parametrical statistical tests, data of sperm volumes were log-transformed using the formula log $(x + 1)$. Sperm volumes are given in $mm^3$. Values are provided as mean ± STD.

ROLE OF SENSILLUM NUMBER AND AEDEAGAL WIDTH. — Each of 25 males was allowed to copulate with one, previously hand-paired female. These pairs were interrupted at the 50th flexion (the point where volume of stored sperm reaches its lowest value; CÓRDOBA-AGUILAR, 1999). The stored sperm volumes in females were measured, the sensilla were counted and the width (in that region that induces the stimulation; see CÓRDOBA-AGUILAR, 2002) of aedeagi measured. The role of sensillum number and ae-

deagal width in predicting remaining sperm was examined using a multiple regression.

NEURAL COMMUNICATION BETWEEN A VAGINAL PLATE AND ITS CORRESPONDING SPER-MATHECA. − A set of 54 previously hand-paired females was kept in envelopes in complete darkness (to reduce the insects' activity) and hand-fed with fruit flies for one day. Females were then randomly allocated to groups of 18 individuals. In the first female group (the "severed" group), the nerves running from the left vaginal plate to the 8th abdominal ganglion were severed as follows. An incision was made at the dorsal portion of the 8th abdominal segment under a dissection microscope. The abdominal tergite was folded back to expose the internal female genitalia. The blackish nerves were easily distinguished from the clear background. The exposed nerves were cut using sharpened scissors and then the tergite was put back. The second female set (the "dissected" group) was treated in the same way but the nerves were left intact while the third set (the "untreated" group) was not dissected at all. The three female groups were kept for one day (under the same conditions as before) after which the experimental manipulation was conducted. On the day after the manipulation, 3 females of the "severed" group, 1 of the "dissected" and 2 of the "untreated" were found dead, so the samples sizes were reduced to 15, 17 and 16 individuals for each group respectively. All females were subjected to copulatory-like stimulation using the same aedeagus. The aedeagus used had its distal head cut off to prevent that the lateral horns could penetrate the spermathecae. The aedeagus was washed, using distilled water, after each treatment to avoid possible "contamination" of ejected sperm from one female to another. The stimulation consisted of 50 in-and-out aedeagal movements for each female during 100 seconds (for a detailed description of this see CÓRDOBA-AGUILAR, 2003). Both, the number of aedeagal movements and duration of this stage are similar to natural matings (CÓRDOBA-AGUILAR, 1999, 2000).

EXPERIMENTAL INCREASE IN THE FLEXION NUMBER TO SIMULATE A PROLONGED SPERM DISPLACEMENT STAGE. − The number of abdominal flexions occurring during stage I (where the sensory stimulation process takes place) is fairly constant. In 1997, an experiment was designed to test if more flexions would cause an increase in sperm ejection from the spermathecae. Each of 10 aedeagi was used to stimulate two different females that had been previously hand-paired. 50 in-and-out aedeagal movements were produced with the first set of females and 80 with the second set over 100 and 160 seconds respectively. Although 80 aedeagal flexions was chosen arbitrarily, it represents a considerable augment (more than 50 %) to a normal copulation. 160 seconds was calculated as an extrapolation from 80 flexions.
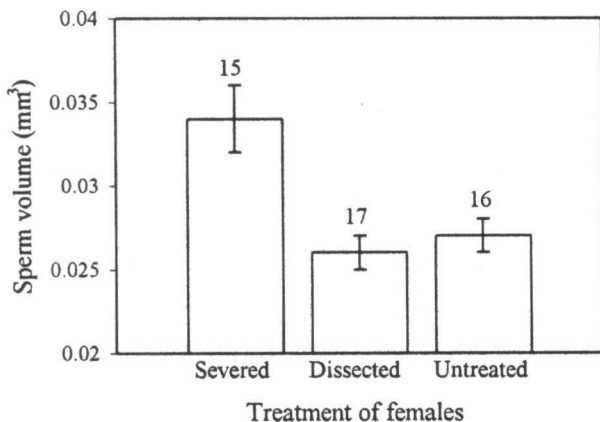


Fig. 1. Volume of sperm (mean ± s.e. bars) remaining in the left spermatheca after the experimental treatment. "Severed" are females whose nerves running from the left vaginal plate to the VIII abdominal ganglion were severed; "Dissected" are females that were dissected but their nerves were left intact; "Untreated" are females that were not dissected. Number of females used per treatment is shown above each bar.

# RESULTS

## ROLE OF SENSILLUM NUMBER AND AEDEAGAL WIDTH

Only aedeagal width ($\beta$ = - 0.322, P < 0.0001) but not sensillum number ($\beta$ = 0.0002, P > 0.05) could predict the amount of sperm remaining in both spermathecae ($F_{2,22}$ = 13.11, $R^2$ = 0.502, P < 0.0001).

## NEURAL COMMUNICATION BETWEEN EACH VAGINAL PLATE AND ITS CORRESPONDING SPERMATHECA

There were significant differences in the volume of sperm stored in the left sperma-theca among the "severed" (0.034 ± 0.006 mm³), the "dissected" (0.026 ± 0.003 mm³) and the "untreated" (0.027 ± 0.004 mm³) groups (ANOVA: $F_{2,45}$ = 13.9, P < 0.0001; Fig. 1) after the treatment. The "severed" group had significantly higher sperm volumes than the other two (Tukey multiple comparison test, P < 0.05).

## EXPERIMENTAL INCREASE IN THE NUMBER OF AEDEAGAL MOVEMENTS

The volume of sperm did not decline significantly when an increment from 50 (0.054 ± 0.006) to 80 (0.053 ± 0.006) aedeagal movements was carried out (paired t-test = 1.69, NS).

# DISCUSSION

Variation in sperm displacement ability has been documented in different insect spe-cies (see, for example, THORNHILL, 1983; SIVA-JOTHY, 1987b; SIVA-JOTHY & TSUBAKI, 1989; OTRONEN, 1990, 1997; OTRONEN & SIVA-JOTHY, 1991; RO-DRÍGUEZ, 1994) but the causes underlying it are unknown. In this paper we exam-ined two questions related to the variation in spermathecal sperm displacement ability in *C. haemorrhoidalis* males.

Recent interest in female-mediated processes during copulation has stimulated re-search on the role played by females in the mechanisms used by males to avoid sperm competition (EBERHARD, 1996). Male *C. haemorrhoidalis* use the aedeagus to in-duce female's ejection of stored sperm (CORDOBA-AGUILAR, 1999). Despite the evidence that variation in sperm displacement ability was related to the width of the aedeagus (CÓRDOBA-AGUILAR, 1999), inter-female variation in sensillum number could also play a role. Contrary to this possible expectation, we found no evidence that the sensillum number had a role but the aedeagal width did. The most parsimonious explanation for the pattern of male consistency is that sperm displacement ability was more reliant on male traits than female genitalic traits. We interpret these results in the context that females use the same "criterion of choice": females will eject high sperm volumes when stimulated with wider aedeagi. The fact that other potentially contrib-

uting variables (male age and status [territorial and nonterritorial], natural duration of the sperm displacement stage and the natural number of aedeagal stimulatory flexions; CÓRDOBA-AGUILAR, 2001) to affect sperm displacement do not explain sperm ejection along with the results presented here, suggest that aedeagal width is the only variable that predict sperm diplacement from the male perspective. Female neural organisation plays its role with the total sensillum number favouring wider aedeagi and with the asymmetry in retaining control over one spermatheca. Linked to this, there are some interesting and obvious questions: what do asymmetrical females gain and why are some females symmetrical (around 20 % in the population; CÓRDOBA-AGUI-LAR, 2003) ? These questions are currently under investigation.

The sensory system that is involved in egg laying and fertilisation in *C. haemorrhoidalis* consists of two vaginal plates with the embedded sensilla, the VIII abdominal ganglion and the two spermathecae (CÓRDOBA-AGUILAR, 2003). We experimentally investigated if there was a control of sperm ejection in each spermatheca by its corresponding vaginal plate by severing nerves. The evidence presented here suggests that a "lateralised" neural communication exists between the vaginal plates and each spermatheca. Despite the fact that the nerves of both plates converge in the VIII abdominal ganglion (CÓRDOBA-AGUILAR, 2003), it appears that the stimulus they communicate separate once they leave this organ to "innervate" the corresponding spermatheca.

The number of copulatory stage I flexions in natural copulations are fairly constant (CÓRDOBA-AGUILAR, 2000). Since the volume of sperm stored in the spermathecae declines gradually with the number of flexions in this stage (CÓRDOBA-AGUILAR, 1999), it is plausible that an increased number of flexions might result in a higher rate of sperm displacement. However, the degree of sperm ejected did not change after experimentally increasing the number of aedeagal flexions suggesting there is a limit on sperm displacement which is reached at around 50 flexions. It is possible that localised spermathecal sperm contractions elicited by sensillum stimulation have resulted in the ejection of the sperm present in the vicinity of those contractions. More flexions may therefore be ineffective as there would be no more sperm to be expelled in those localised regions. This hypothesis is supported by the observation that spermathecal sperm disposition is not homogenous at the end of stage I (CÓRDOBA-AGUILAR, 1999). According to this reasoning, wide and narrow aedeagi differ in the number of sensilla they are able to reach and stimulate.

Genitalic characters are very diverse in form and, possibly, function (EBERHARD, 1985, 1996). It has been suggested that post-copulatory sexual selection may be responsible for the elaboration and diversity of male genitalia (EBERHARD, 1985, 1996; ARNQVIST, 1998; CORDERO RIVERA et al., 2004). Some recent evidence has provided support to this view (RODRÍGUEZ, 1995; ARNQVIST et al., 1997; ARNQVIST & THORNHILL, 1998; ARNQVIST & DANIELSSON, 1999; TADLER, 1999, CÓRDOBA-AGUILAR, 1999, 2002; CORDERO RIVERA et al., 2004). However, some of these studies have suffered from a lack of a mechanistic explanation of how genitalic characters are favoured. This has impeded a thorough understanding of the evolu-

tion of genitalic specialisation. The research in *C. haemorrhoidalis* shows how much can be gained in this respect once the interaction of both sexes during copulation has been elucidated.

## ACKNOWLEDGEMENTS

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