

## Limited spermathecal sperm removal ability in the damselfly *Hypolestes trinitatis* (Gundlach) (Odonata: Megapodagrionidae)

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It has been hypothesized that sperm removal ability in male Odonata (damselflies and dragonflies) has promoted sexual conflict over the sperm stored in the reproductive tract of the female. Although there is evidence supporting this hypothesis, most studies have been conducted in a small number of species from specific families. We explored sperm removal ability in the Antillean Megapodagrionidae, *Hypolestes trinitatis* through examination of specialized structures on the genital ligula (“penis”) and through measurement of sperm volumes stored in the sperm storage organs (bursa copulatrix and spermathecae) at different stages of the copula. Males removed sperm from the bursa, but not from the spermathecae. The penis has four finger-like terminal processes covered by spines which could contribute to sperm removal. Given the width of the penile processes, males could introduce them into the spermathecae to remove sperm; however this does not seem to occur. A possible explanation for the sperm removal pattern of *H. trinitatis* could be that the penile processes are prevented to reach the sperm stored due to their position in relation to the spermathecae during the copulation.

**Keywords:** sperm competition; sperm removal; sexual conflict; Odonata; dragonfly; *Hypolestes*

### Introduction

Sexual selection is an often strong type of natural selection that arises from competition over mates (Andersson & Iwasa, 1996), but the concept can be generalized to include the fitness effects due to differences between the sexes in optimal strategies for survival and reproduction, thus leading to a situation in which the genes have to face different selective scenarios depending on sex (Carranza, 2009). It is clearly not limited to the pre-copulatory interval, as initially stated by Darwin (1871), but it can continue inside the female’s reproductive tract in the form of competition between the sperm of various males to fertilize the ova (Parker, 1970), or as cryptic female choice, a series of female mechanisms to bias paternity on the basis of male syn- and/or post-copulatory courtship (Eberhard, 1996).

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Sperm competition is a widespread phenomenon among animals with internal fertilization (Birkhead & Møller, 1992; Gomendio et al., 1998; Simmons, 2001). Sperm competition promotes the evolution of anatomical, physiological and behavioral mechanisms in males that increase their fertilization success. Examples of such mechanisms are the production of higher volumes of ejaculate (Birkhead and Møller, 1992) and spermatozoa with improved motility (Gomendio & Roldán, 1991; Møller, 1988), the regulation of the amount of inseminated sperm according to both male density (Simmons et al., 1993) and reproductive status of the female (Jivoff, 1997), and the transfer of mating plugs to isolate the rival sperm (Diesel, 1990) and/or to avoid female remating (Contreras-Garduño et al., 2006).

Direct removal of sperm from the female reproductive tract by means of male specialized genital structures is a widespread mechanism in Odonata (Córdoba-Aguilar & Cordero-Rivera, 2008; Córdoba-Aguilar et al., 2003), although it has been observed in several other orders of insects, such as the Dermaptera, Orthoptera and Coleoptera (Haubruge et al., 1999; Helversen & Helversen, 1991; Kamimura, 2000; Ono et al., 1989; Yokoi, 1990), and even in a cuttlefish (Wada et al., 2005), which suggests this might be a widespread phenomenon. Before sperm transference is achieved, male odonates remove rival ejaculates stored by the female from previous matings (Cordero & Miller, 1992; Waage, 1979). However, this is not the only sperm displacement mechanism that has evolved in odonates. Alternatively males inseminate multiple times during a single copulation, probably to outnumber rival sperm or flush it out (Cordero et al., 1995), reposition stored sperm in the female tract by pushing it to places far from the fertilization site (Siva-Jothy, 1988; Waage, 1982) or induce sperm ejection via sensory stimulation of the female (Córdoba-Aguilar, 1999) (reviewed by Cordero-Rivera & Córdoba-Aguilar, 2010; Córdoba-Aguilar et al., 2003).

Male and female genitalia in the Odonata have been coevolving antagonistically by sexual selection, with the sperm stored in the female sperm storage organs (SSOs) as the center of the conflict (Cordero-Rivera & Córdoba-Aguilar, 2010; Córdoba-Aguilar et al., 2003). The SSOs are sac-like structures that usually comprise the bursa copulatrix and one or two spermathecae. The shape of the SSOs is variable and generally corresponds with the penis morphology and the mechanism the male uses to avoid sperm competition (Córdoba-Aguilar, 2003a). Males usually remove the sperm from the bursa copulatrix; however the penis is sometimes prevented from reaching the spermathecae by a narrow spermathecal duct (Córdoba-Aguilar, 1999), or due to lack of specialized structures such as spines on the lateral horns of the penis (Cordero et al., 1995). This mechanical impediment imposed by the female has been interpreted as a counteradaptation to avoid the male's control over the fertilization process (Cordero-Rivera et al., 2004).

Sexual conflict arises when the evolutionary interests, e.g. the decisions on the fertilization process, differ between individuals of the two sexes (Parker, 1979, 2006). The statement that there is sexual conflict over the sperm stored in the spermathecae derives its empirical support from intensive studies in a few genera of some specific families. If we are to make generalizations about the evolution of the genitalia in the odonates we need data on copulatory mechanisms in a wider range of species and families (Córdoba-Aguilar et al., 2003).

The term Megapodagrionidae is employed to classify a group of tropical zygopterans, which however do not constitute a monophyletic family according to cladistic analyses that combine molecular and morphological data (Bybee et al., 2008), nor according to nuclear DNA markers (Dumont et al., 2010). The biology of this family has been poorly studied (see Corbet, 2004, for a review of the biology of Odonata). We only have a few studies, mainly limited to the description of their reproductive behavior (González-Soriano & Verdugo-Garza, 1982; González-Soriano & Córdoba-Aguilar, 2003; Torres-Cambas & Fonseca-Rodríguez, 2009; Romo-Beltrán et al., 2009), but there is no information about the sperm displacement mechanisms.

The aim of this study is to explore male sperm removal ability in *H. trinitatis* through examination of specialized structures on the penis and through measurement of sperm volumes stored in the SSOs at different stages of the copula.

## Materials and methods

As in other odonates, copulation in *H. trinitatis* is divided in two stages (Torres-Cambas & Fonseca-Rodríguez, 2009) with the first probably dedicated to sperm removal and the second to sperm transfer. We used two experimental sets of females to test the hypothesis of male sperm removal ability in this species. The first group (hereinafter “interrupted”,  $n = 8$ ) was allowed to complete one copulation, mated with a second male and interrupted at the end of stage I, presumably before insemination (Miller & Miller, 1981). The second set of females (hereinafter “one copulation”,  $n = 9$ ) was allowed to mate only once. Both groups were immediately stored in 70% ethanol after copulation. All the samples were independent since each male was used for only one copulation.

The specimens were collected at Dos Bocas stream, 12 km N of Santiago de Cuba, eastern Cuba (20° 5' 36.2" N, 75° 45' 44.4" W). This is a small mountain headwater stream of  $c. 2$  km length and 5–10 m width, situated at 200 m asl.

To measure stored sperm volumes, the sperm storage organs were dissected and compressed to a uniform thickness of 0.2 mm with an insect pin situated between the coverslip and the slide. Subsequently, the area occupied by the sperm was measured from images captured with a video camera attached to a compound microscope and analyzed with UTHSCSA ImageTool 3.0 software (available at <ftp://maxrad6.uthscsa.edu>). The sperm volume was calculated by multiplying the area by the uniform thickness (Cordero & Miller, 1992).

If males are able to remove sperm at stage I, it should be expected that sperm volume stored by the interrupted females will be lower than the volume stored by females after completing one copulation. A permutation test for two independent populations was used to test the null hypothesis of equality between treatments. This test is suitable when normality is not met and the sample size is small (Siegel & Castellan, 1995). The calculations were performed with StatXact-4 version 4.0.1 (Cytel Software Corporation, 1999 Cambridge, MA, USA. URL is <http://www.cytel.com/Software/StatXact.aspx>).

Our observations indicated that the penis bears four terminal processes at its tip. To test whether such processes physically enter and presumably remove the spermathecal stored sperm, we measured the width of the four terminal processes in their widest region ( $n = 9$ ) and the width of the base of the spermathecae ( $n = 9$ ), with the software ImageTool from images captured at the compound microscope (Figure 1). Since the lateral processes become narrower at their tips we also measured the width at their first third. If the processes were narrower than the base of the spermathecae it should be expected that they can access the spermathecal stored sperm and remove it. The precision in all measurements was 0.01 mm.

The results are expressed as the mean  $\pm$  standard error and the significance level used was 0.05.

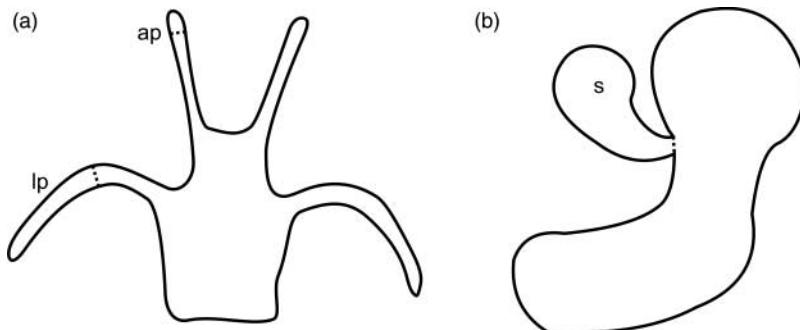


Figure 1. Genital structures measured in both sexes of *Hypolestes trinitatis*. (a): extended penis head showing the lateral processes (lp) and the apical processes (ap). (b): female genitalia with the spermathecae denoted by (s). The dashed lines indicate the sites where measurements were performed.

## Results

The penis of *H. trinitatis* is composed by four finger-like terminal processes (Figure 2a). Two of these structures are straight, roughly cylindrical and are located distally in the so-called penis head. The other two processes are curved, compressed and are located lateral to the straight ones (Figure 2a). The terminal processes, as well as the exterior surface of the sperm channel, are covered by spines, which could trap the sperm mass and help during sperm removal (Figure 2a, b, c, d, e).

Female sperm storage organs in *H. trinitatis* consist of a bursa copulatrix, which communicates ventrally with the vagina and dorsally with a tubular and apically wide spermatheca of  $0.36 \pm 0.026$  mm ( $n = 9$ ) in length (Figure 2f, g). Two sclerotized structures, the vaginal plates, are anterior to the union of the vagina with the bursa and have several embedded sensilla (Figure 2h). The number of such sensilla was  $72 \pm 3.4$  and there were no significant differences in the number of sensilla between left ( $34 \pm 1.8$ ,  $n = 11$ ) and right ( $36 \pm 1.9$ ,  $n = 11$ ) plates (Wilcoxon sign rank test for two paired samples:  $T = 36.5$ ;  $p = 0.875$ ).

The mean sperm volume in the bursa of the one copulation group ( $0.036 \pm 0.004$  mm<sup>3</sup>,  $n = 9$ ) was twice that in the interrupted group ( $0.017 \pm 0.004$  mm<sup>3</sup>,  $n = 8$ ) (permutation test:  $T = 0.32$ ;  $p = 0.0023$ ). However no difference was observed in the spermathecal sperm volume between treatments (one copulation:  $0.008 \pm 0.001$  mm<sup>3</sup>,  $n = 8$ ; interrupted:  $0.009 \pm 0.001$  mm<sup>3</sup>,  $n = 7$ ; permutation test  $T = 0.064$ ,  $p = 0.4311$ ).

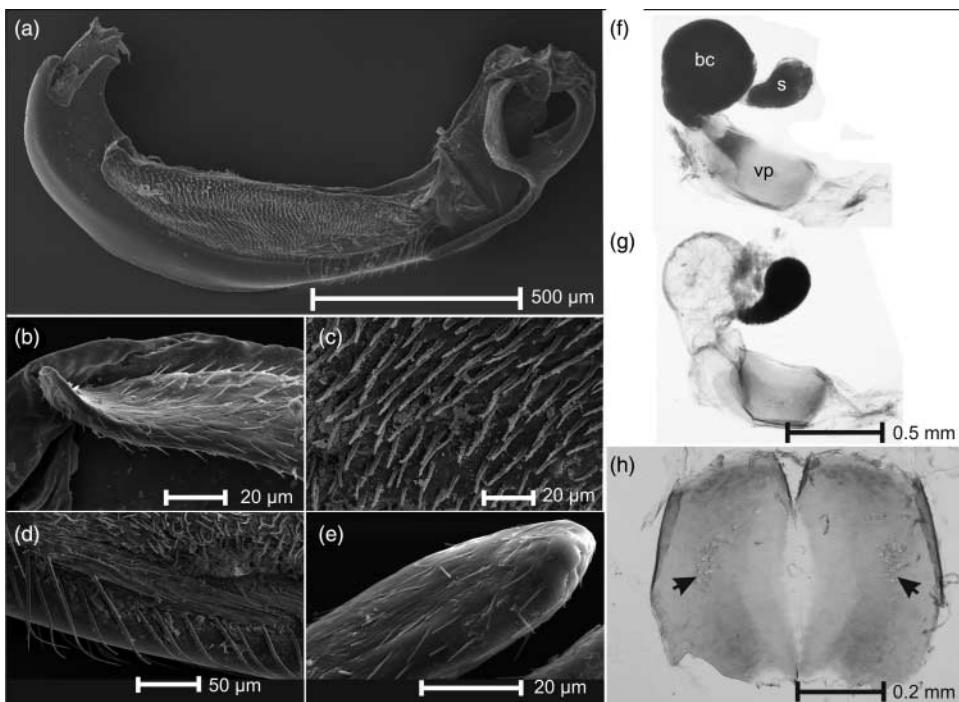


Figure 2. Male genitalia in *Hypolestes trinitatis*: (a) lateral view of the penis showing the finger-like terminal processes; (b) spines on a lateral process, on the spermatic channel (c, d) and on an apical process that could facilitate sperm removal. (f) Genitalia from a female after one copulation showing both the bursa (bc) and the spermatheca (s) full of sperm and the vaginal plates (vp). (g) Genitalia from a female interrupted after stage I: the sperm has been removed from the bursa, but not from the spermatheca. (h) Vaginal plates showing embedded sensilla (arrows).

The lateral processes did not differ in width (left:  $0.05 \pm 0.002$  mm,  $n = 9$ ; right:  $0.05 \pm 0.002$  mm,  $n = 9$ ), nor did the apical ones (left:  $0.03 \pm 0.001$  mm,  $n = 9$ , left:  $0.03 \pm 0.001$ ,  $n = 9$ ). The apical processes were narrower than the base of the spermathecae (spermathecal width:  $0.06 \pm 0.004$  mm; one-tailed Mann–Whitney U test = 0;  $p < 0.0001$ ). The lateral processes in their widest region were not narrower than the base of the spermathecae (one-tailed Mann–Whitney U test = 23.5;  $p = 0.053$ ), but the two structures did not differ in width (two-tailed Mann–Whitney U test = 23.5;  $p = 0.106$ ). However the lateral processes at their first third were narrower than the base of the spermathecae (width of the lateral processes at their first third:  $0.04 \pm 0.002$ , one-tailed Mann–Whitney U test = 6.0;  $p = 0.0007$ ).

## Discussion

We have found that *H. trinitatis* males have specialized structures in their penis, likely used to remove sperm. Nevertheless, their ability to remove sperm from the spermatheca, the organ used for long-term storage of sperm (Nakahara & Tsubaki, 2007) seems limited.

There is great variability in the penis morphology of odonates. In Zygoptera, four groups can be recognized according to the penis head morphology (Cordero-Rivera & Córdoba-Aguilar, 2010): with two large and flexible flagella; spoon-like with a conspicuous flexible joint; wide head with two to four distal processes; and without flexible joint and with a variable number of lobules. The penis of *Hypolestes trinitatis*, with four distal processes, is included in the third group.

The bursa of *H. trinitatis* is rounded and tubular, similar to those of the platynemidids, *Platynemis acutipennis* (Córdoba-Aguilar et al., 2003) and *P. latipes* (Uhía & Cordero-Rivera, 2005). However, odonates vary in shape and volumetric capacity of these organs, in the presence or absence of spermathecae and the number of its ducts. For example, some Lestidae (e.g. Uhía and Cordero-Rivera, 2005; Waage, 1982) and the Platynemididae, *Copera annulata* (Watanabe and Adachi, 1987), lack spermathecae; *Lestes viridis* (Uhía & Cordero-Rivera, 2005) has two, reduced and without communication between them, and *Calopteryx haemorrhoidalis* has two spermathecal ducts (Córdoba-Aguilar, 1999), although 1% of females have only one (Cordero-Rivera et al., 2004).

The primary function of the sensilla in the vaginal plates in Odonata is to detect the presence of the egg at fertilization (Córdoba-Aguilar, 2003b). The number of such sensilla ranges from 47 to 88 in Calopterygidae and is asymmetrically distributed between plates. This asymmetry has been interpreted as a female adaptation to independently control the contraction intensity of each spermathecae, and bias the use of the sperm (Córdoba-Aguilar, 2003a). Contrary to Calopterygidae, *H. trinitatis* has only one spermathecae; therefore, as might be expected, the distribution of sensilla was not biased.

Differences in the sperm volume of the bursa between treatments confirm sperm removal in *H. trinitatis*. In odonates, a correspondence has been found between the functional morphology of the penis and the morphology of the bursa and spermathecae (Córdoba-Aguilar et al., 2003). Therefore, we could expect that distal processes in the penis of *H. trinitatis* are used to remove the sperm from the spermatheca. However, these structures apparently do not have access to the spermatheca, since the sperm volume in this organ was similar for one-copulation females and for those interrupted at the end of stage I. This limited ability to remove spermathecal sperm has been previously found in some other odonates, such as the coenagrionids *Ischnura elegans* (Miller, 1987), and *Ceriagrion tenellum* (Andrés & Cordero-Rivera, 2000), and the calopterygids *Mnais pruinosa* (Siva-Jothy & Tsubaki, 1989; Tsuchiya & Hayashi, 2008), *Calopteryx xanthostoma* (Córdoba-Aguilar, 2002; Hooper & Siva-Jothy, 1996) and *Hetaerina cruentata* (Córdoba-Aguilar, 2002). It has been proposed that this is the result of sexual conflict over control of fertilization,

where females regain this control by impeding males from removing sperm from these organs (Córdoba-Aguilar & Cordero-Rivera, 2008). In this context, it is interesting to note that males of *Calopteryx haemorrhoidalis asturica* from Pontevedra, north-western Spain do not have access to the spermathecae, but the ejection of the rival sperm is induced by stimulation of the vaginal sensilla (Córdoba-Aguilar, 1999). In a population of the same species (*C. h. haemorrhoidalis*) from Frosinone, central Italy, nevertheless, males are able to access the spermathecae (Cordero-Rivera et al., 2004).

The apical processes of the penis of *H. trinitatis* were narrower than the base of the spermatheca, so there was no evidence for the presence of physical impediments to these processes to remove the spermathecal sperm. The lateral processes, moreover, were narrower than the base of the spermathecae, although just at their tips, which indicates that at least a segment of these processes might have access to the sperm stored by the female. However the spermathecal sperm displacement did not occur despite the supposed lack of physical impediment. These results, apparently contradictory, coincide with those from Miller (1987) in *Ischnura elegans*. In this species the penis head ends in two long and thin flagella-like structures, but males do not remove the sperm from the spermathecae. On the contrary, in *I. graellsii* with a similar genital morphology, sperm is removed from the spermathecae (Cordero & Miller, 1992). A possible explanation for the sperm removal pattern of *H. trinitatis* could be that the penile processes are prevented to reach the sperm stored due to their position in relation to the spermathecae during the copulation. In the zygopteran the position the penis head has during copulation could be important for male sperm removal ability, since males probably do not control the movements of the penis head, because this organ seems to lack muscles and tracheae (Tillyard, 1917).

The retention of sperm in the spermathecae represents an opportunity for females to choose cryptically and bias paternity (Cordero-Rivera & Córdoba-Aguilar, 2010; Uhía and Cordero-Rivera, 2005). If this is true, genital evolution in *H. trinitatis* should not be explained just by the sperm competition hypothesis, but the point of view of the female should be considered. Our study shows that sperm removal has also evolved in the Megapodagrionidae, but more studies of this large and probably artificial family are needed.

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