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SPERM DISPLACEMENT IN THE DAMSELFLY, *XANTHAGRION ERYTHRONEURUM* (ZYGOPTERA: COENAGRIONIDAE) - VARIANCE IN FEMALE SPERM COUNT AND GENITAL MORPHOLOGY

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ABSTRACT

Sperm competition was examined in the non-territorial damselfly, Xanthagrion erythroneurum *(Selys) (Odonata: Zygoptera: Coenagrionidae) in a small freshwater lake (Forrestdale Lake reserve), which is close to city Perth, Western Australia. Mating pairs were collected along the shores of lake in 3 categories: precopula, interrupted copula and postcopula. Evidence of sperm removal in* X. erythroneurum *was found from two sources: counts of the number of sperm and penis/female genitalia morphology. Females captured during copulation had fewer sperm in their storage organ than pre- and post-copula females. These results suggest that male* X. erythroneurum *can remove rival sperm from a female's storage organ during copulation.*

The morphology of the penis shows that the distal appendage of the penis is a recurved flap-like structure covered with small spines. These structures suggest that the male scoops sperm from the bursa copulatrix before or during deposition of its own sperm. After removing the sperm from the previous matings, new sperm is discharged through a channel which opens on the tip of penis.

INTRODUCTION

Female insects store sperm in their sperm storage organ, the spermatheca, and where the female mates with more than one male the question arises as to whose sperm fertilizes the eggs? Parker[4] , in his paper on sperm competition pointed out that male reproductive success and not necessary the number of times he mates depended on how many of his sperm are successful in fertilizing eggs. Given this degree of selection in polyandrous species, males will be expected to possess traits that promote the success of their sperm over that of rivals.

In many insects, sperm competition occurs within the spermatheca, and in these cases it is often the last sperm to be placed in the genital tracts that achieve first fertilisation. However, a more drastic method is to prevent the other male's sperm entering the sperm storage organ or to remove rival sperm from the storage organ before determing its own. Preventing a second male's sperm from entering the spermatheca can be achieved through sperm plugs, while the use of spoon-like processes, attached to the penis can remove sperm from a previous mating. In both cases males often ensure fertilisation by protracting copulation or by guarding the female once it is inseminated[7].

Sperm competition by sperm removal has been shown in several odonate species. The first evidence for this was found in *Calopteryx maculata* by Waage[8] who showed how a male could remove almost all the sperm of its rival before depositing its own. In other species only some of the sperm from previous matings is removed before the second male replaces the sperm with its own[1, 5, 6, 9]. In such cases, sperm precedence will result from mechanisms whereby the last male to mate achieves most fertilisations.

The mechanism of sperm removal involves in odonates highly specialised morphology of the distal segment of the male's penis[3]. Siva-Jothy[5] suggested that the barbed flagellum of the penis, which fits into the narrow ducts of the spermathecae, was used to remove sperm from females during copulation in *Orthetrum cancellatum* (Anisoptera). However, the distal segment of the penis of *Calopteryx maculata* (Zygoptera) has a flexible scoop-like flap, and carries two horn-like appendages. These structures are used to remove sperm from the bursa copulatrix by the scoop-like flap and from the spermatheca by the horned appendages[8]. Waage also showed how the structure of the penis in males closely matches the structure of the female genitalia. In *Lestes vigilax* (Zygoptera) the penis is much more simple in that there are no horns or flaps and as a result sperm removal is much less complete[9].

This study examines sperm removal by male *Xanthagrion erythroneurum*. But as Waage[10] points out techniques for establishing the mechanism *in situ* particulary, for zygopterans are extremely difficult and so two approaches are used in this study. First, we use deductive reasoning based on genital morphology employing both the insights of Waage[10] and Miller[3]. Second, we conduct a series of field observations based on sperm counts of mated and un-mated females.

By studying *Lestes vigilax* , Waage[9] inferred sperm removal by showing that the volume of sperm in the female changed during copulation. A simple hypothesis was that during sperm removal, sperm volume should be lowered. By measuring sperm volume within the bursa copulatrix during interrupted copula he assumed that as the female withdraws her genitalia, late in the copulation cycle, sperm volume should be large, and this sperm should comprise that of the last male.

MATERIALS AND METHODS

Animals were collected along the shores of a small freshwater lake close to the city of Perth, Western Australia (Forrestdale Lake reserve) during early summer (November/ December).

Mating pairs were placed in vials (4 x 11 cm), at which time they broke off mating. They were then transferred live to the laboratory for dissection. Once in the laboratory females were killed by squeezing the thorax after which the bursa copulatrix and spermatheca were collected by nipping the abdomen with fine forceps. These organs were placed in a small cone shaped vial (1.5 ml in volume) filled with 0.2 ml of "dragonfly solution"[2]. The tissues were ruptured by a few strokes of a small plastic stick which dispersed the sperm throughout the solution. Sperm were mixed and homogenized with 1 ml syringe before a few drops were transferred to a blood haemacytometer. The number of sperm in a given volume of haemacytometer (0.9 mm^3) was counted and sub-samples of it were measured. Sperm of *X. erythroneurum* is 0.052 ± 0.003 (mean ± se) mm long (personal observe). Two estimates of the number of sperm were made from each sample from which a mean was calculated.

Sperm counts were made in the storage organ of females captured before, during and after copulation. These categories were defined as (i) "precopula", when both sexes are in tandem and before forming the wheel posture, (ii) "interrupted copula", when mating pairs are in a wheel position (pairs were sampled at different intervals through the copulation), (iii) "postcopula", when mating pairs have broken the wheel formation and both fly to an oviposition site in tandem. Eleven pairs were caught and mating duration was measured in 8 cases.

For morphological studies, both female gentital tract and the male penis were dissected from freshly killed insects and prepared by standard techniques for Scanning Electron Microscopy (SEM).

RESULTS AND DISCUSSION

Sperm counts

If there was no sperm displacement by males those females interrupted during copulation should have the same number, or more sperm than pre-copula females. Females captured during copulation had a lower sperm count in their dissected genitalia than pre- and post-copula females (pre-copular: mean 747 ± 69 (SE), n = 11; post-copular mean 994 ± 58 (SE), n = 11; $F = 4.46$, $P < 0.05$), which strongly suggests that male *X. erythroneurum* remove rival sperm from the female's storage organ during copulation.

If sperm replacement was taking place then the number of sperm should initially decline to a minimum and then rise to post-copula levels. This change in sperm number should best be illustrated by the sperm content of the bursa copulatrix and spermathecae of females sampled during copulation. Sperm number was lower during interrupted copulation (mean 278 ± 46 (SE), n = 11) and was significantly lower than pre- and post-copula levels (pre-copula vs interrupted copula; $F = 16.02$, $P < 0.05$: post-copula vs interrupted copula; $F = 37.41$, $P < 0.05$). Fig 1 shows the changes in sperm count and stage of the copulation cycle.

Ideally, the relationship between sperm number and the point at which copulation is interrupted should follow a quadratic function. But based on a sample size of 8 interrupted copulations we were unable to demonstrate this relationship (Fig 2). We provide three simple explanations why we were unable to achieve this. Firstly, it was likely due to the small sample size. Secondly, the number of sperm present in the female genitalia, prior to copulation, was extremely variable between individuals (range: 392-902 sperm/sample). A third reason could have been the contribution to sperm count variation by inter-individual variability in duration of copulation. Resolving these objections is far from easy and indeed, in field captured females may be impossible to achieve.

Males are clearly delivering sperm, however, the three females sampled post-oviposition showed a decline in sperm number (mean number of sperm was 267±65 (SE)). Some of this sperm loss will be due to fertilisation but some may be due to the passage of the egg through the vagina/oviduct. In our observations, sperm counts were made on total sperm content of the female genitalia (spermatheca, bursa copulatrix and vagina) and without dissection of each component we have no idea as to just how much sperm rests within the vagina/oviduct. We presume that sperm still resides within the female storage organ and tracts after oviposition.

Morphology

A second line of evidence to suggest that sperm displacement occurs in *X. erythroneurum* comes from the morphology of the penis and female reproductive tracts. The distal appendage of the penis is shaped as paired recurved flap-like structure (Fig 3A) where the flaps are covered with small backwardly projecting spines on the inner surface (Fig 3B). The general morphology of the penis in *X. erythroneurum* appears most similar to that of *Calopteryx* [10]. Waage suggests that structures equivalent to these flaps in *C. maculata* are used by the male to scoop sperm from the bursa copulatrix of female so allowing the male to deposit new sperm within the female's genital tract[8,10]. This does not appear to be the most likely copulation for *X. erythroneurum* which varies with such a scooping action in a number of important ways.

The structure of the female genital opening and associated structures, the vagina, bursa copulatrix, single spermatheca and ascending oviducts, are similar to that of the genus *Enallagma*[10]. From histological and SEM examination the vagina and bursa copulatrix appear much smaller than the penis. However, such a discrepancy may be because the soft tissues of the female's

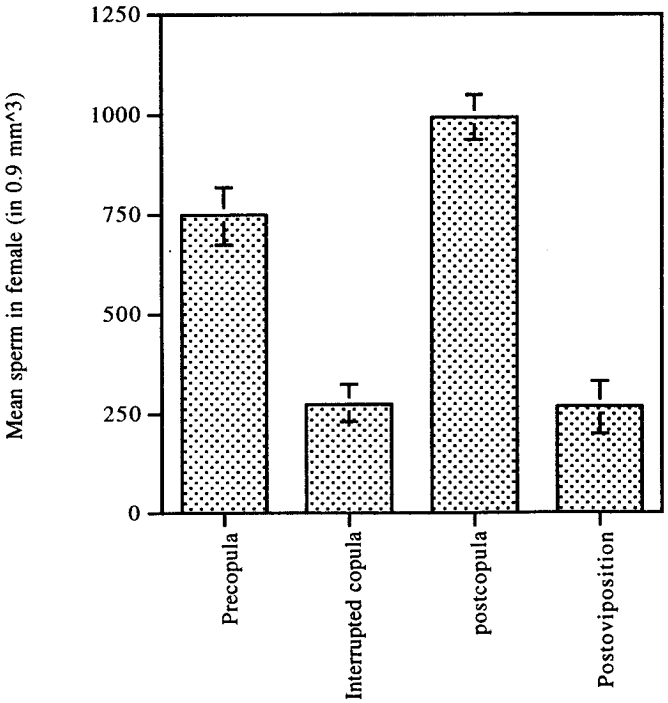

Fig.1. Comparison between the mean number of sperm (standard error bars) from the female's storage organ of precopula (n=11), interrupted copula (n=11), postcopula (n=11) and postoviposition (n=3).

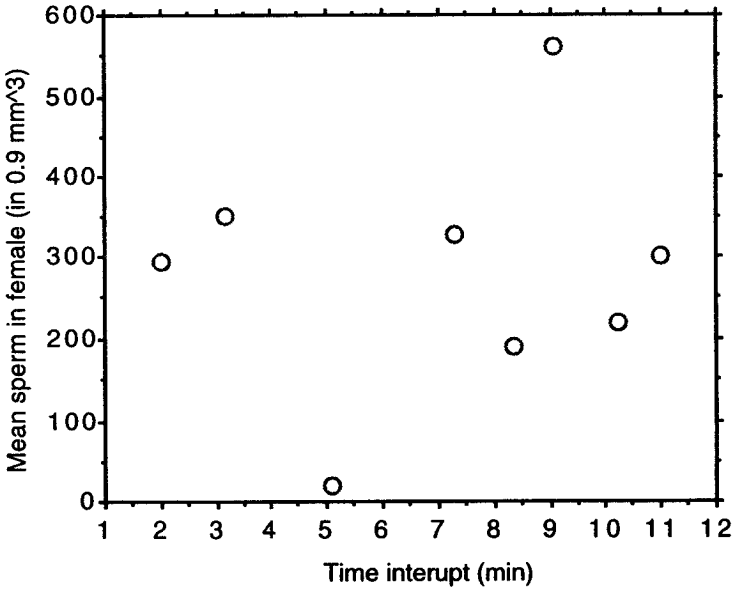

Fig.2. Number of sperm stored in the sperm storage organs of females captured during copulation (interrupted copula) (means, n=8).

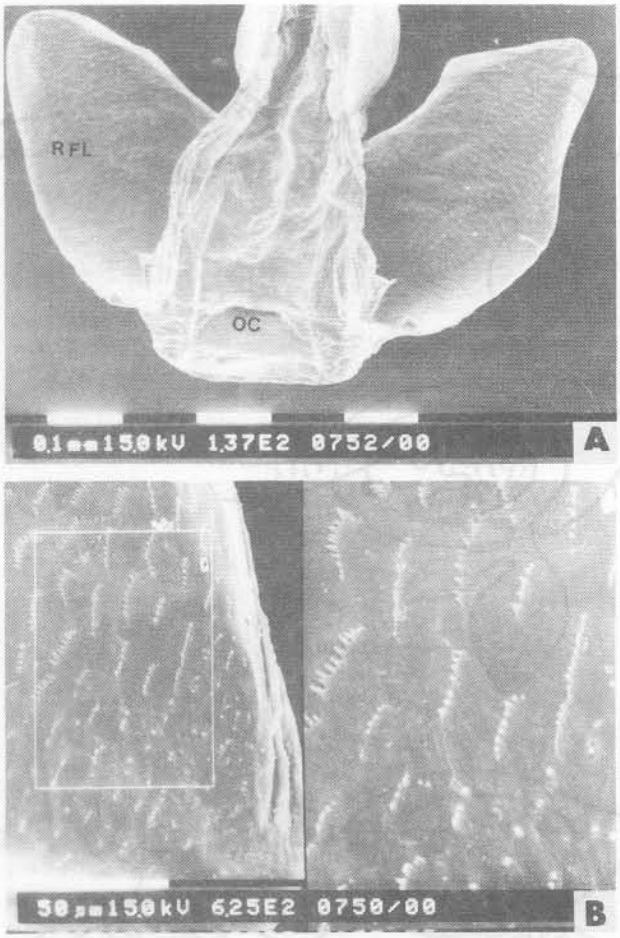

Fig.3. Scanning electron micrograph of the distal appendage male penis (A) and spines cover distal appendage of penis (B) of *X. erythroneurum*. OC=Opening channel of sperm, RFL= Recurved flap like.

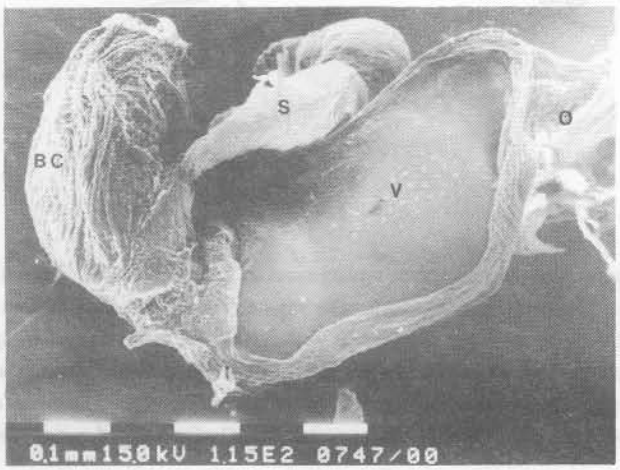

Fig.4. Scanning electron micrograph of the female genitalia of *X. erythroneurum*. BC=Bursa copulatrix, O=Oviduct, S=Spermatheca, V=Vagina.

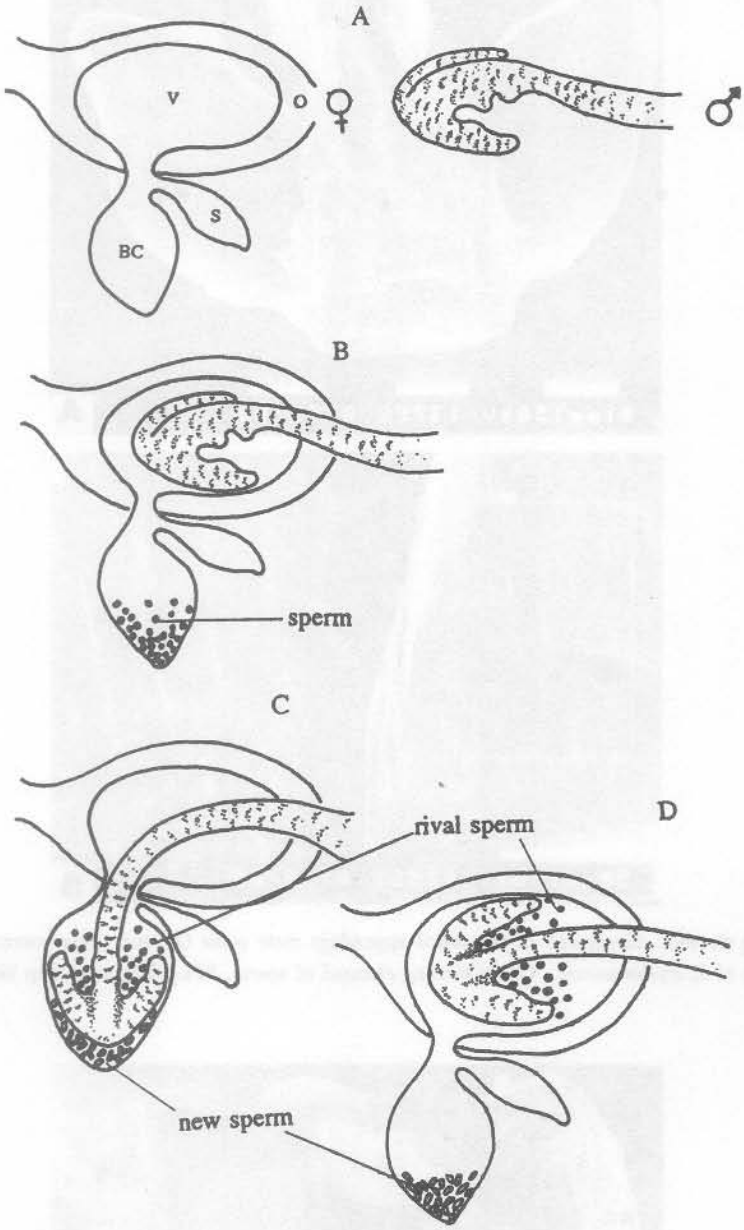

Fig.5. Mechanism of sperm removal and sperm replacement in *X. erythroneurum*. Lateral view of male and female genitalia (Fig. A). The penis is inserted into the vagina (Fig. B) and then pressed into the bursa copulatrix where the paired flaps open. When the penis is withdrawn, sperm are trapped by spines on the under surface of the flaps which then act as valves. As the sperm from the previous matings are removed new sperm is discharged through a channel opening on the tip of penis (Fig. C). The penis is withdrawn into vagina removing the rival sperm (Fig. D). BC=Bursa copulatrix, O=Opening of vagina, S=Spermatheca, V=Vagina.

reproductive tracts are more susceptible to shrinkage in preparation than is the hard tissue of the male penis. The female reproductive parts are undoubtedly highly elastic, both allowing the entry of the penis and ensuring that the tract closes around the inserted penis (Fig 4). Conceivably, the retracted flaps behind the penis will allow the female to place its genital opening over the penis, or for the male to insert the penis into the vagina. (It is not clear as to which sex has the active role in intromision.) However, once within the vagina, the penis would be pressed into the bursa copulatrix, a still smaller, though presumably equally elastic structure to the vagina.

We suggest that the flaps do not act as "active" spoons, removing sperm but rather any sperm within the vagina and bursa copulatrix from a previous mating is pressurised around the penis, effectively displaced by the movement and bulk of the penis head. Once in position, the recurved flaps could then act as "valves" within the vagina/bursa copulatrix. In addition the minute recurved spines could act as a series of combs, so preventing sperm moving toward the vacated space ahead of the penis. As the penis is retracted the existing sperm would be removed and the cavity filled by new sperm delivered by the inseminating male (Fig 5).

This process is clearly different from that proposed by Waage[10] for the *Ischnura sp.* where he describes two recurved hooks at the end of the penis which conceivably enter the bursa and spermatheca. That described for *Enallagma* sp. has a single hook which also can enter the bursa and *Calypteryx* sp. which has a paired spoon matching the branched spermatheca. In all these species Waage proposes an active process by spoon/hook-like structures. We suggest that in *X. erythroneurum* sperm is displaced and actively removed by the withdrawing penis.

But in *X. erythroneurum* the problem remains as to what is the fate of the sperm remaining in the spermatheca: there is no obvious way by which the male can retrieve sperm from this organ. Arguably if oviposition occurs immediately after insemination sperm, first used for the fertilisation of eggs, will be that remaining in the genital tracts and the bursa. If this is the case then we may predict that mate guarding to the point of oviposition will be under strong selection as a mechanism that ensures the most recently injected sperm is used rather than that residing in the spermatheca.

There is no field evidence for females ovipositing without males in this species, but if they were to do this, as is the case in many Zygoptera, then first mated males may regain an advantage of fertilisation from their residing in the spermatheca: sperm trapped in the bursa and the vagina would presumably be used during the initial oviposition following insemination. Our observations on changes in sperm count during copulation would indicate that there are still sperm present post-oviposition (Fig 1) and it would be instructive to know if these sperm were those of the first male.

Many questions are unresolved by this study, and one of significance to the insect's mating system is the control the male has over penis withdrawal. In other words which sex controls copula duration? The recurved spines may have evolved, initially as a trapping mechanism to ensure that the female, once mated could not interrupt copulation. Conceivably the flaps or spines of other species could act as a spring clamp mechanism. But such an hypothesis suggests that the "spoons" are there as female fixing structures and not, at least primarily, as sperm removal organs. However, once selection begins to operate through sperm competition structures such as the recurved spines or recurved flaps could now evolve as sperm displacement mechanisms.

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บทคัดย่อ

การแข่งขันของสเปิร์มในการแย่งผสมกับไข่ของตัวเมียในแมลงปอเข็มที่ไม่สร้างอาณาเขตครอบครองชนิด *Xanthagrion erythoneurum* (Selys) (Odonata: Zygoptera: Ceonagrionidae) ที่อาศัยอยู่ตามแหล่งน้ำจืดของเมืองเพิร์ท รัฐออสเตรเลียตะวันตก ประเทศออสเตรเลียในการศึกษาได้ทำการจับตัวอย่างของคู่ตัวผู้และตัวเมียที่กำลังผสมกันอยู่ตามริมชายฝั่งของแหล่งน้ำซึ่งได้แบ่งออกเป็น 3 กลุ่มคือ คู่ผสมก่อนมีการผสม (precopula) คู่ผสมที่กำลังผสม และถูกแยกก่อนที่จะผสมเสร็จ (interrupted copula) คู่ผสมที่เสร็จสิ้นการผสมแล้ว (postcopula) หลักฐานของการกำจัดสเปิร์มของคู่แข่งมาจาก 2 แหล่งคือ การนับจำนวนของสเปิร์มและสัณฐานของอวัยวะสืบพันธุ์ของทั้งสองเพศ จากการนับจำนวนของสเปิร์มของคู่ผสมในตัวเมีย พบว่าจำนวนสเปิร์มของตัวเมียขณะที่ถูกแยกก่อนที่จะผสมเสร็จมีจำนวนสเปิร์มน้อยกว่าตัวเมียที่ก่อนเข้าผสมและตัวเมียหลังจากที่ผสมเสร็จแล้ว จากผลที่ได้แสดงให้เห็นว่าตัวผู้สามารถกำจัดสเปิร์มคู่แข่งที่เก็บสะสมไว้ในอวัยวะเก็บสเปิร์มที่อยู่ในตัวเมียขณะที่กำลังผสมกันอยู่

หลักฐานทางสัณฐานของอวัยวะสืบพันธุ์ของของเพศผู้และเพศเมีย พบว่าอวัยวะของตัวผู้มีลักษณะคล้ายหัวลูกศรที่พับเก็บส่วนหัวได้ และปกคลุมด้วยขนแข็งเล็กๆ โครงสร้างเช่นนี้ทำให้ตัวผู้สามารถกวาดสเปิร์มที่อยู่ในอวัยวะเก็บสเปิร์มของตัวเมีย (bursa copulatrix) ในระยะก่อนที่จะเข้าผสมและขณะที่กำลังผสมอยู่ ก่อนที่จะปล่อยสเปิร์มของตนเองเข้าแทนที่ โดยสเปิร์มจะปล่อยผ่านช่องเปิดตรงปลายของอวัยวะสืบพันธุ์