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## Underlying sperm precedence pattern in the millipede *Centrobolus inscriptus* (Attems, 1928) (Diplopoda, Pachybolidae)

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### Abstract

Three scenarios exist for sperm precedence; first-male, last-male and no order effect. I replicated the  $H^{3+}$  radiolabel technique to show sperm precedence patterns in the arthropod *Centrobolus inscriptus* (Attems, 1928). Male and female morphometric parameters illustrated a clear sexual dimorphism. Disintegration rates (d.p.m.) from immediate dissections of once-mated females were higher than females dissected after 24 hours (L (0) » L (24)). A significant difference between mean d.p.m. values occurred in double mating (L-UL vs. UL-L). D.p.m. values were higher when second mating followed immediately after the first (UL-L (0) > UL-L (24) and L-UL (0) » L-UL (24)). D.p.m. from L mating were significantly lower than those of UL-L mating but not then L-UL double mating. There was a reduction of up to 57% in favour of the second male over 24 hours in *C. inscriptus*.

**Keywords:** Arthropod, mate-guarding, precedence, sex, size, sperm precedence

### 1. Introduction

Sperm competition can be selected in the re-mating interval <sup>[1]</sup>. Recent evidence for Mechanisms of sperm competition is provided in arthropods include the millipede *Centrobolus inscriptus* <sup>[8]</sup>, the fly *Drosophila melanogaster* <sup>[11]</sup>, the squid *Euprymna tasmanica* <sup>[12]</sup> and the moth *Ephestia kuehniella* <sup>[15]</sup>. Male-mediated mechanisms can be reduced down to simple processes of removal, repositioning, displacement, and mixing and because the operational role of anyone of these processes is both species- and sex-specific the costs and benefits accrued by individual males and females must be quantified separately. Millipedes are polygynandrous and exemplify sperm competition which can be explained as sperm precedence patterns for the competitors <sup>[8]</sup>. Three scenarios exist for sperm precedence, namely, first-male precedence, last-male precedence, and no order effect, although this may change with additional mating <sup>[8]</sup>.

When any of the numerous methods used to quantify sperm precedence *e. g.* sterile male method, genetic marking or some forms of DNA fingerprinting are used to calculate sperm precedence, the females' influence on the storage and use of sperm may go undetected. Here the  $H^{3+}$  radiolabel technique was implemented in order to account for the role of females. In the present article I replicated the radiolabel technique to show sperm precedence patterns in the diplopod *C. inscriptus*. *C. inscriptus* belongs to the forest genus of millipedes in the Order Spirobolida found along the eastern coast of southern Africa <sup>[2]</sup>. It has evolved sperm competition strategies through either conflict of interest or co-operation <sup>[16]</sup>.

It was questioned if there is sperm competition during the re-mating interval and sperm precedence becomes one male favoured within 24 hours.

### 2. Materials and Methods

#### 2.1 Observations

*Centrobolus inscriptus* (Attems, 1928) were collected from Zululand (Twin streams farm, Mtunzini), where they inhabited indigenous coastal forest (28°59'13.4"S, 31°43'41.3"E). Live specimens of each sex were transported to the laboratory where conditions were kept under a constant regime of 25°C temperature; 70% relative humidity; 12:12 hrs light-dark cycle. Food was provided in the form of fresh vegetables *ad libitum*. Individuals had unknown mating histories and unisex groups were housed in plastic containers containing moist vermiculite ( $\pm 5$ cm deep) for 10 days before commencing the first mating experiments.

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## 2.2 Data analysis

Three measurements were taken for all individuals once copula pairs had disengaged; body mass (accurate to 0.01 g), body length (mm), and dorsal tergite width (mm). Morphometric data was tested for normality before comparing males and females using a Student's t-test.

## 2.3 Experimental layout

Animals were placed into glass mating arenas (30 X 22 X 22 cm) for single, double, and artificially-terminated mating with females. Four combinations of double mating possible: L-UL (0); L-UL (24); UL-L (0); UL-L (24). In *C. inscriptus* an experimental protocol was based on the Tritiated [methyl-3H] thymidine radioisotope labelling technique <sup>[1]</sup>. The radioactivity of ejaculates inseminated into a female spermatheca, by labelled males, were quantified as the volume of *labelled* (L.) ejaculate present in the female spermatheca.  $V_2$  was calculated as a surrogate variable for  $P_2$  is obtained.

## 2.4 Scanning electron microscopy (SEM)

Male accessory intromittent organs (gonopods) were removed from dead males by hand and prepared for SEM. Gonopods were viewed under a Cambridge S200 SEM.

## 2.5 Statistical analysis

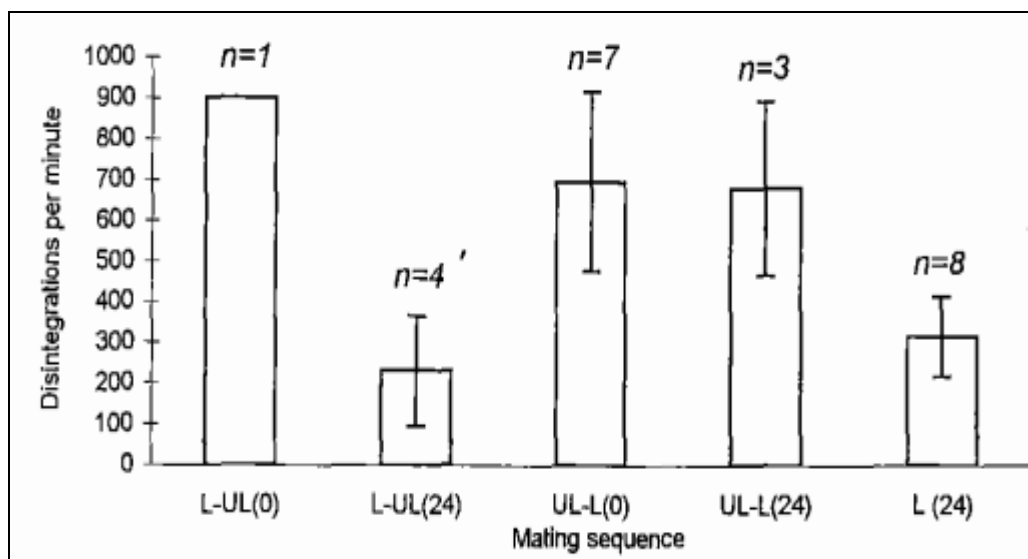
Statistical analyses were performed using Statgraphics (version 6.0). Morphometric data were tested for normality

before comparing males and females using a Student's t-test. Two-tailed Mann-Whitney u-tests were employed as non-parametric analogues to t-tests. Pearson's correlation coefficients were used to analyse any other relationships.

## 3. Results

*C. inscriptus* is clearly sexually dimorphic and shows length and width-based variability. Male body mass explains a significant percentage of body length ( $R^2=0.65$ , d. f. = 54,  $p<0.005$ ) and dorsal tergite width ( $R^2=0.09$ , d.f.=54,  $P<0.005$ ). The same is true for female body length ( $R^2=0.79$ , d.f.=39,  $p<0.005$ ) and dorsal tergite width ( $R^2=0.58$ , d.f.=39  $p<0.005$ ). Coefficients of variation for mass differed between the sexes ( $F=0.47345$ , d.f.=55, 40,  $P = 0.01025$ ) indicating directional sexual selection.

Disintegration rates (d.p.m.) from immediate dissections of once-mated females were higher than from females dissected after 24 hours (L (0) »L (24)). There was a significant difference between mean d.p.m. values of double mating (L-UL vs. UL-L: Two-tailed Mann-Whitney U-test (unpaired),  $U=2.4$ ,  $n=15$ ,  $p=0.001$ ). D.p.m. values were also higher when second mating followed immediately after the first (UL-L (0) >UL-L (24) and L-UL (0)>L-UL (24)). D.p.m. rates from L mating were significantly lower than those of UL-L mating ( $U=-2.6$ ,  $n=13$ ,  $p=0.005$ ) but not to L-UL double mating ( $U=0.45$ ,  $n=14$ ,  $p=0.65$ ). Without the interval  $V_2=43.66$  and with the interval  $V_2=74.59$ .



**Fig 1:** Bar graph showing the amount of radiolabelled ejaculate recovered from the spermatheca of females used in single and double mating. UL, unlabelled; L, labelled.

When the experiment was replicated significant differences between total d.p.m values were again found (Figure 2:  $H = 30.67$ , d.f.= 5,  $P = 0.00001$ ). In effect there is first male precedence without an interval ( $V_2= 0.21$ ). When there is a

delay of 24 hours between successive mating, precedence reversed (UL-L(24) > L-UL (24):  $z = -2.59$ ,  $n = 8,14$ ,  $P = 0.01$ ) to  $V^2 = 0.77$ .

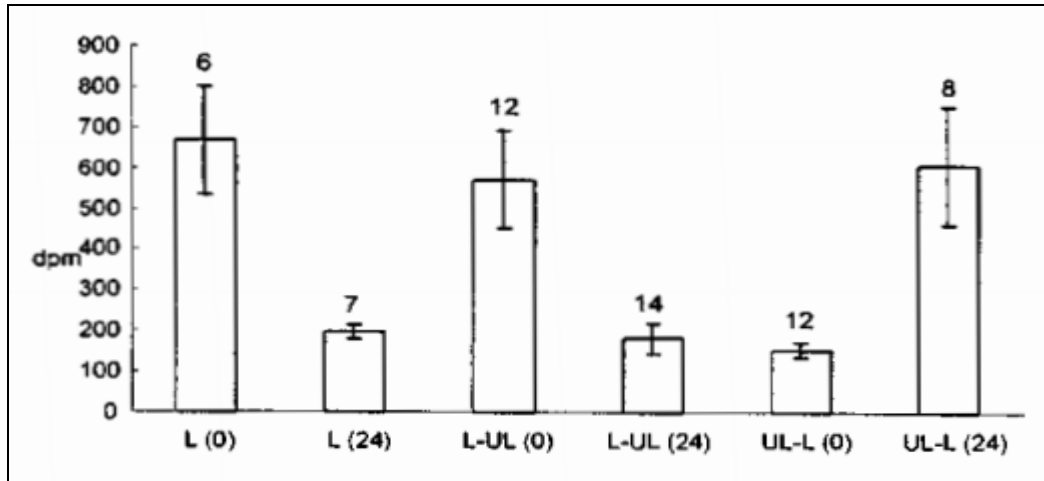


Fig 2: Replication of Figure 1.

Individual components of *C. inscriptus* gonopods have been identified (Figure 3). The overall gonopod structure of *C. inscriptus* is relatively simple and apparent from the telopodites (Figure 4). The telopodite plate bears pitted-spines (Figure 5) which have been viewed on male genitalic structure before.

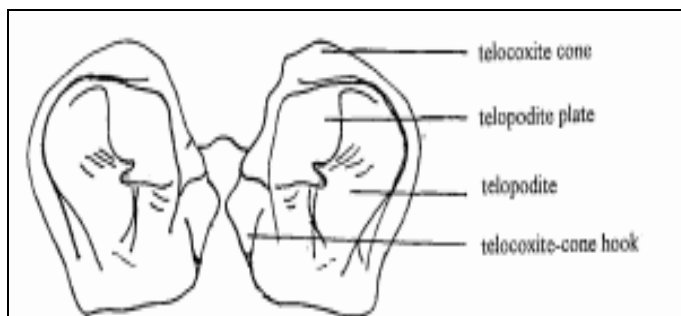


Fig 3: Line drawing of an aboral view of *Centrobolus inscriptus* gonopods labelling the different structural components.

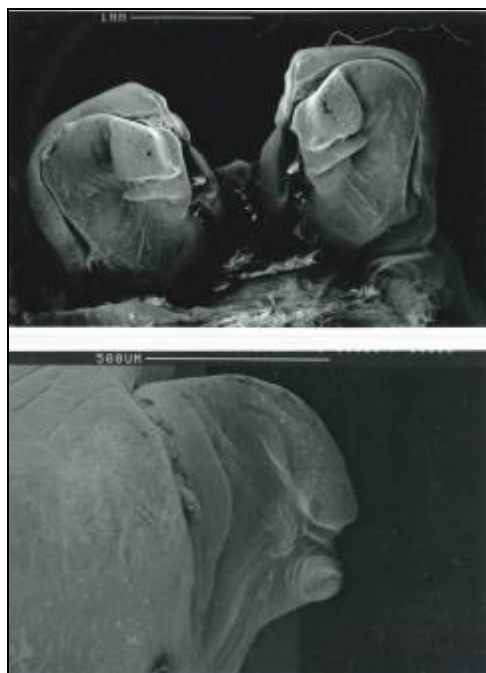


Fig 4: Scanning electron micrograph showing the structural features of *Centrobolus inscriptus* male gonopods (accessory intromittent organs); a. aboral view showing the overall structure; b. distal region of coxite.

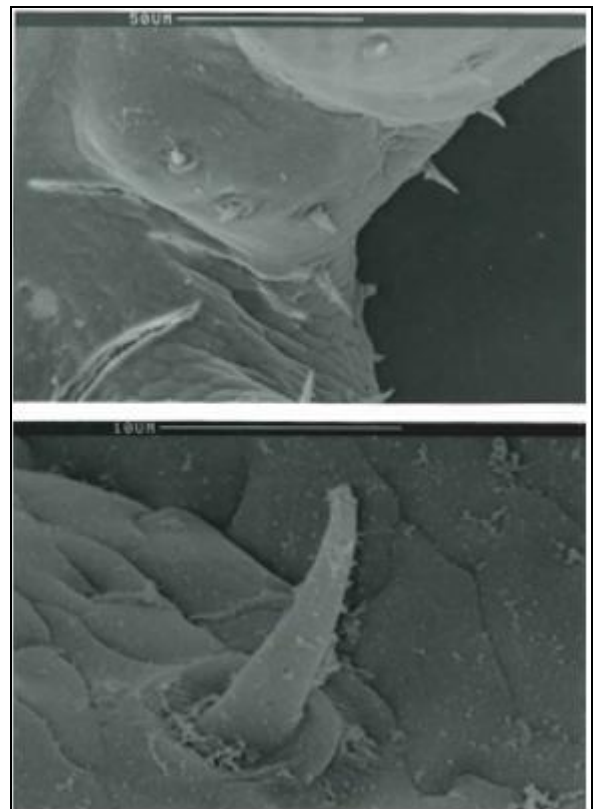


Fig 5: Scanning electron micrograph showing the surface features of *Centrobolus inscriptus* gonopods; a. distally located spines of the telopodite plate; b. Magnified view of a single spine situated in a pit-like base.

#### 4. Discussion

*C. inscriptus* is sexually dimorphic showing length- and width-based variability, factors common to members of the worm-like millipedes which were correlated with mass [3-6, 9, 10]. There was a reduction of up to 57% in favour of the second male over 24 hours which may develop with the number of mating an individual has experienced. Size-assortative mating was found in the laboratory and morphometric trends suggest males may be adapted for increased mobility and ability to locate females. Males that mate last prolong copulations in accordance with their body size.

Three scenarios for sperm precedence were tested *i. e.* first-male precedence, last-male precedence, and no order effect. In *C. inscriptus* second-male precedence is evident from the

lower labelled ejaculate volumes present when a 24-h delay is imposed between mating. This equates to a decline in  $V_2$  over time seen in millipedes where second-male precedence is female mediated with a 24-hour interval<sup>[8]</sup>. This also shows successive mating produces opposite patterns on ejaculate volume and selection on female re-mating interval is influenced by male sperm competition strategies<sup>[1, 13]</sup>. Body size and reproductive performance are related in *C. inscriptus* because second-male sperm precedence is achieved by those individuals that prolong mate-guarding relative to their own body mass.

Extensive telopodite retraction-release cycles are associated with prolonged copulations which is a behaviour which will only provide benefits in the presence of rival ejaculates *i.e.* when males copulate with a previously mated female. Last-male precedence may be more effective in assuring paternity when a female has already mated with two males<sup>[15]</sup>. Thus, last-male precedence is affected when sex-ratios are male biased<sup>[7]</sup>, because of the increased probability that a female has already mated.

In millipedes (*Antichiropus variabilis*) genital morphology influences paternity success and here spinescent hairs occurring on the distal regions of the gonopod coxites which are thought to displace sperm through a 'scouring' effect<sup>[14]</sup>. The spines on the gonopod telopodite plates in *C. inscriptus* may have other functions including female choice, sperm displacement, and male 'anchoring'. Spinescent hairs in *C. inscriptus* are suggested to maybe function in female choice as the probability that a female will mate promiscuously is positively correlated to genitalic complexity and copulation behaviour<sup>[12]</sup>. The less complex telopodites of *C. inscriptus* may necessitate a longer retraction-release cycle in order to achieve the equivalent effect of the more complex telopodites in other millipedes such as *D. uncinatus*. Prolonged copulation durations in *C. inscriptus* is a consequence of simple gonopods being less effective than complex gonopods in sperm mixing and 'repositioners' having relatively short copulations (seconds) compared with these 'movers' (minutes-hours).

As millipedes have aflagellate sperm it makes sense the entire ejaculate was considered the unit of selection. Thus these results are surprising as they could consider the evolution of anisogamy consistent with cooperation rather than conflict of interests<sup>[16]</sup>. An optimization strategy of sperm competition within 24 hours is suggested for *C. inscriptus*<sup>[17]</sup>.

## 5. Conclusion

Radiolabelling of millipede ejaculates successfully shows female-mediated second-male sperm precedence while male-specialized genital structures function in sperm redistribution. Second males benefit from mate-guarding prolonged copulation in accordance with body size to counter sperm competition.

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