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Instantaneous insemination in the millipede *Centrobolus inscriptus* (Spirobolida: Trigoniulidae) determined by artificially-terminated mating

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Abstract
Male millipedes may control the duration of copulation and maintain genital contact with females in relation to the intensity of sperm competition. Post-insemination associations were considered in arthropods and the timing of insemination in the millipede *Centrobolus inscriptus* calculated by artificially-terminated radiolabelled mating. Copula pairs were separated after 1, 2, 3, 4, 5, 10, 20, and 30 minutes of copulation. Ejaculate volumes inseminated did not correlate significantly with the manipulated copulation durations (r = -0.35, df = 8, P = 0.35). High (*ca.* 2500 dpm) and low values in the first 5 minutes evidenced "instantaneous-insemination" and self-sperm displacement. Instantaneous insemination was demonstrated using artificially terminated mating in *Centrobolus inscriptus* by showing no relationship between ejaculate volume and copulation duration except for the high and low volumes at the beginning of mating when the male loads and seats the gonopods before adaptive mate-guarding by prolonged copulation.

Keywords: Arthropoda; Centrobolus inscriptus; Diplopoda; postinsemination; mate-guarding

1. Introduction

Mate guarding is known in the Arthropod classes Diplopoda [1-14, 58-59]; Araneae [44, 47-48]; Acari [45-46, 61, 65]; Crustacea [49-50, 56-57, 60, 62]; Scorpiones [52]; Opiliones [53]; and Insecta, orders Orthoptera [31, 51], Odonata [26, 34, 35], Phasmida [36], Lepidoptera [21, 37], Diptera [30, 38], Coleoptera [19, 20, 22, 27, 29, 39, 40, 63-64], Hymenoptera [28, 41], Hemiptera [15-18, 23-25, 42-43]. Post-insemination associations and the mate-guarding hypothesis has been previously reviewed for Insecta [32]. Millipedes (Class: Diplopoda) have highly polygynandrous mating systems that are very complex because males and females can mate multiply and prolong copulations with maintained genital contact where males control the duration of copulation and maintain genital contact with females in relation to the intensity of sperm competition [1-4]. The instantaneous timing of insemination has been detected using radiolabeled ejaculates in *Alloporus uncinatus* (Spirostreptida: Sprirostreptidae), followed by the adaptive mate-guarding phase with maintained genital contact [13]. The collection of sperm at the bottom of the spermathecae from the beginning of insemination with time after the start of copulation indicated prolonged copulation was the adaptive mate-guarding phase [13]. The objective of the present study is to determine the timing of insemination in the millipede *Centrobolus inscriptus*, replicating the artificially-terminated radio-labelled mating experiments which were performed in *A. uncinatus* [13], to test if prolonged genital contact is the adaptive mate-guarding phase.

2. Materials and Methods

Millipedes were hand collected (1996-1998) from indigenous coastal forest at Twin Streams Farm, Mtunzini, South Africa (28°55'S, 31 °45'E). Live specimens of each sex were transported to Cape Town and kept at 25°C temperature; 70% relative humidity; 12:12 hours light-dark cycle. Food was provided in the form of fresh vegetables *ad libitum*. Unisex groups were housed in plastic containers containing moist vermiculite (± 5 cm deep) before the mating experiments commenced. The radioisotope labelling technique allowed two types of ejaculates to be discerned [11]. A Hamilton syringe was used to inject 50 uL aliquots of tritiated [*methyl*] thymidine (85 Ci I mmol, Amersham, UK) between the tergites of the 10th and 11th diplosegments of individual males (L). A second class of males did not receive the treatment and were left unlabelled males (UL). Females were killed in ethyl-acetate jars after their last copulation and the paired sperm storage organs were dissected out under a magnifying lens

(5X). Left and right spermathecae were placed in separate 7ml scintillation vials and vortexed for 30s with 0.l ml concentrated HCl to promote rapid tissue homogenisation [12]. Acid was neutralised with 0.l ml 5M NaOH prior to adding 3.5 ml scintillation fluid (Scintillator 299, Packford). The volume of labelled ejaculate present in the female spermathecae was quantified in disintegrations per minute (dpm) of radioisotope using a 1600 scintillation counter (low count reject = 0; dpm multiplier = 1). Thus dpm values were used as volumetric indications of the labelled ejaculate present in the female spermathecae. Radiolabelled males were used in artificially-terminated matings to elucidate the timing of insemination [12]. Copula pairs were separated after 1, 2, 3, 4, 5, 10, 20, and 30 minutes of copulation. Statistical analyses were performed using Statgraphics (version 6.0). The non-parametric nature of disintegration counts [11], coupled with small sample sizes, did not allow data to be normally transformed. Pearson’s correlation coefficients were used to analyse the relationships between ejaculate volume, copulation time and body mass.

3. Results

Ejaculate volumes (Figure 1) inseminated did not correlate significantly with the manipulated copulation durations (r = -0.35, df = 8, P = 0.35) but the first few data points indicate very high volumes (*ca.* 2500 dpm) deposited within the first five minutes interspersed with the range of lower volumes. Male body mass was not related to ejaculate volume (r = 0. 54, df = 8, p = 0. 13) and was not related to copulation duration (r = -0.64, df = 8, P = 0.06).

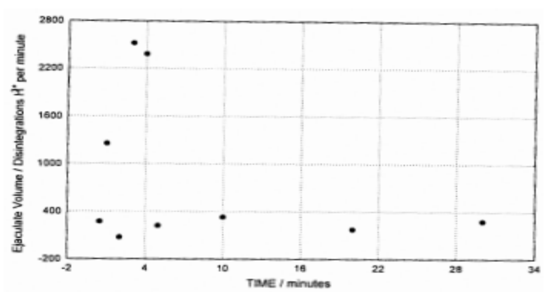

Fig 1: The ejaculate volumes inseminated by radiolabelled males of *Centrobolus inscriptus* when matings were terminated at different stages.

4. Discussion

The lack of correlation between ejaculate volume inseminated and manipulated copulation duration evidenced "instantaneous-insemination" during which time the male loads and seats the gonopods before engaging in the adaptive mate-guarding phase [12]. This was supported by high and low volumes of ejaculate in the first five minutes of copulation supporting self-sperm displacement and matches the predicted results found in the spirostreptid *A. uncinatus* [13]. The absence of an allometric relationship here supports the previous findings in millipedes. This is an interesting result and probably indicates this is the situation in all Julid millipedes where there is a post-insemination association. It was critical to discern between the different stages or phases of copulation in order to show mate-guarding as an adaptation to counter sperm-competition through the integration of pre- or syn-copulatory and post-copulatory events [33].

5. Conclusion

Instantaneous insemination was demonstrated using artificially terminated mating in *Centrobolus inscriptus* by showing no relationship between ejaculate volume and copulation duration except for high and low volumes at the beginning of mating when the male loads and seats the gonopods before adaptive mate-guarding by prolonged copulation.

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