

MATING DYNAMICS OF SOUTH AFRICAN FOREST MILLIPEDES

CENTROBOLUS (DIPLOPODA: PACHYBOLIDAE)



BY

MARK I. COOPER

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MATING DYNAMICS OF SOUTH AFRICAN FOREST
MILLIPEDES

CENTROBOLENUS (DIPLOPODA : PACHYBOLIDAE)

BY

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Dedicated to Ken I. Cooper (1945-1996) & Helen P. Cooper for the 'good genes',

to being one with the "cryptofauna" (R. F. Lawrence 1897-1987)
beneath the forest canopies,

to you the reader,

mu

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ABSTRACT / Cooper, M. I. 1998. *Mating dynamics in South African forest millipedes Centrobolus* (Diplopoda: Pachybolidae). Masters Thesis, Department of Zoology, University of Cape Town, Rondebosch 7701, South Africa, (vi) + 142 pp.

This study focuses on the mating dynamics of South African forest millipedes *Centrobolus* (Diplopoda: Spirobolida: Pachybolidae). The main objectives were to investigate the two most common mechanisms of sexual selection, sperm competition and cryptic female choice. The approach was to (1) quantify sexual dimorphism and find the selection pressures operating on the sexes, (2) determine the functional significance of male and female genitalia, (3) understand why there should be a conflict of sexual interests in prolonged copulations, and (4) resolve the mechanisms of sperm competition and cryptic female choice by comparing male mating strategies and sperm precedence to female mating strategies and sperm usage. All hypotheses tested in this thesis are centered on whether there is a conflict of interests between the sexes.

Sexual size dimorphism (SSD) was formulated according to the cylindrical *bauplan* of the juliform millipede where individual body sizes were calculated as cylindrical volumes. At the intraspecific level there is disruptive selection operating on body size. The quantitative resolution of SSD on body size for 18 species produced an (hyper) allometric coefficient of 1.3 corroborating Rensch's rule. The most plausible functional hypotheses for 'expanding' females and 'elongating' males are fecundity and sexual selection, respectively. Male tarsal pads are grasping structures that hold females during copulation.

Sexual selection has shaped both male and female anatomies that function in mating. The primary sex organs are anterior (i.e. progoneate): females have a pair of vulva on diplosegment II and males a pair of penes on diplosegment III, in addition to a pair of accessory intromittent organs (gonopods). Scanning electron microscopy (SEM) and histological techniques were used to view these structures. The typical two-paired spirobolidan gonopods of four congeners are species-specific with complexities indicating rapid divergence. This warrants the gonopods as test models for elaborate genitalia hypotheses: lock-and-key, pleiotropism, genitalic recognition, mechanical conflict of interests, cryptic female choice. Potential structures of sperm displacement are recognizable. The upper female reproductive tract was dissected to reveal a pair of bursae that are conservative in structure across the same four species. The architecture shows a single spermatheca separate from the insemination duct and oviduct, each with a set of muscles allowing female control over temporary sperm storage in the insemination duct and permanent sperm storage in the spermatheca. A complex structure, the 'pincushion', is associated with a sensory network that may send messages from the external bursa, via receptors on the bursa, to the muscles of the internal bursa. Biomechanics of the genitalia were reconstructed by the freeze-fixation technique. Posterior gonopods inflate and expand giving the distal regions a two-fold reach into the temporary sperm stores. Males cannot directly access the spermatheca but may affect the distribution of ejaculate in the temporary sperm store. It is debatable whether males can stimulate female musculature, access the oviduct and simulate egg release. The potential decoupling of insemination, fertilization and ovulation has consequences on the coadaptation between male and female genitalia.

Behavioural sequences were described by freeze-framing video footage. Precopulatory struggles lasting up to 10 minutes were characterized by the female coiling up as when responding to predators. Males coerced females into mating by uncoiling them out of this position. Three stimuli produced by the male were candidates of copulatory courtship: antennation, glandular secretion and the hydraulic sexual collar. Female size correlates with copulation duration. The large intraspecific variance in copulation duration indicates that there is a conflict of sexual interests for controlling its termination. Interspecific variance in copulation duration also produces significant trends. In species with highly prolonged copulations (e.g. *C. inscriptus*, 170.0 ± 49.3 min.) the male coiled around the female producing a large 'sexual collar' with hydraulic movements in the region of genital contact. In species with shorter copulations (e.g. *C. ruber*, 39.8 ± 13.2 min.) the pair copulated in parallel and had no apparent sexual collar. Copulatory 'triplets' and 'clusters' of mating pairs, those accompanied by additional males that are always unsuccessful at physical take-overs, indicates that intense male-male competition is manifest as sperm competition.

The functional significance of prolonged copulations and the determinants of sperm usage patterns were investigated using a H^{3+} radiolabel to trace male ejaculates through mating sequences. Single matings of females revealed that the spermathecae were volumetrically limited in their capacity to store ejaculates deposited by treated males. Artificially terminated matings demonstrated that the timing of insemination was early and that there is self-sperm displacement. Differential declines in ejaculate stored over the 24-hour postmating interval suggest adaptive sperm dumping and possibly also cryptic choice by females. In double mating sequences, the volumetric precedence of the second male ejaculate over that of the first ($V_2 = 77\%$) takes place when temporary sperm stores are empty. The patterns of rival-sperm and self-sperm displacement, together with female sperm dumping indicate that there is cryptic female choice. The relationship between genitalic complexity, control of copulation and sexual conflict of interests were investigated in two sympatric species. The operational sex ratio is considered a determinant of sexual conflict of interests.

Polygynandrous mating systems place intense selection pressure on males to maximize copulation numbers and duration, and females to perform a type endurance rivalry to balance the costs. Hence endearing male and enduring female mating strategies are the end product of sperm competition and cryptic female choice in *Centrobolus*.

Key words: millipedes, sexual dimorphism, functional genitalia, prolonged copulation, cryptic female choice, sperm competition, conflict of interests.

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1.

General introduction

Theoretical background

Originally, sexual selection was conceived as operating by two distinct processes of intrasexual selection and intersexual selection (Darwin 1871). Intrasexual processes were explained using competition theories, where the mate is a limited resource: usually it is male-male competition for females. Intersexual processes involved explaining the basis for an individual of the limited sex choosing or selecting amongst a range of individuals of the opposite sex. Later, the distinction between the two processes became modified into male-male competition and epigamic selection because all sexual selection was considered intrasexual (Huxley 1938). Further major theoretical changes took place, one of which was to show how the strength of sexual selection could be measured as offspring production relative to mating success (Bateman 1948, Trivers 1972). Another was based upon how the operational sex ratio (OSR) is a major determinant of potential reproductive rates in males and females (Emlen & Oring 1977, Clutton-Brock & Parker 1992). Today, with competition over mates being the unifying aspect of all sexual selection, as many as seven different mechanisms of sexual selection are recognised from the empirical evidence (Andersson & Iwasa 1996): scrambles, endurance rivalry, contests, coercion, infanticide, sperm competition, mate choice.

Sperm competition and mate choice have the wealth of empirical evidence, thanks to studies on gametes and the processes that take place during the span of copulation. Sperm competition is the manifestation of male-male competition whereby rival males compete

for fertilizations rather than matings *per se* (Parker 1970, Stockley 1997). Cryptic female choice involves selection for elaborate male genitalia, copulatory courtship and postcopulatory products (Thornhill 1983, Eberhard 1996). The dynamics within a mating system may be an evolutionary stable balance between these two mechanisms or the outcome of a conflict of interests between the sexes (Smith 1984, Choe & Crespi 1997).

The dungfly *Scatophaga stercoraria* is a paradigm animal in investigations of sperm competition (Parker 1970, 1984, Parker *et al.* 1993). Ever since males were found to prolong the duration of copulation, similar strategies have been identified in many other taxa. Prolonged copulation is a behavioural strategy for males to counter sperm competition so as to guard their own sperm and increase the probability of fertilisation. The most supporting evidence is illustrated in the damselflies and dragonflies where the male genitalia perform a dual function in sperm displacement and sperm transfer (Waage 1979, Finke *et al.* 1997). Different forms of sperm storage, and the sperm storage organs themselves, determine the use of the remaining rivals' sperm in fertilization (Walker 1980, Eberhard 1996). The range of adaptations that counter sperm competition and stimulate cryptic female choice are well known in the Vertebrates, especially the birds (Class: Aves) (Birkhead & Møller 1992), and in the Invertebrates, especially the insects (Class: Insecta) and spiders (Class: Arachnida) (Choe & Crespi 1997), yet experiments and comparisons with lesser known taxa such as millipedes (Class: Diplopoda) are only just beginning.

Millipedes and the pertinence of empirical evidence

Previous studies of the behavioural ecology of millipedes in southern Africa have revealed how dynamic mating behaviours have evolved, through male-male competition and female choice. Sequences of manipulation experiments have demonstrated that copulation duration

becomes increasingly prolonged when sex ratios are male biased (Telford & Dangerfield 1990*a,b*). The millipede *Alloporus uncinatus* Attems, 1926 (Diplopoda: Spirostreptidae) was discovered emerging *en masse* following the rains in the riparian forests of Zimbabwe (S. R. Telford, *pers. comm.*). It was suggested that these seasonal aggregations of individuals were related to both feeding and mating (Dangerfield & Telford 1991, 1993). Although the feeding explanation holds true for *Alloporus uncinatus*, new questions on how and why individuals emerge in swarms, primarily to mate and not to feed, require studies on the mating dynamics of divergent millipede taxa.

In millipedes, prolonged copulation is an adaptive mate-guarding strategy assuring paternity. This form of *in copula* mate guarding involves the males maintaining genital contact, e.g. *A. uncinatus* (Telford & Dangerfield 1994), and by gaining control of the copulation duration they are successful in reducing sperm competition. The dynamics and functional morphology of these male genitalia were studied together with their conspecific female genitalia and compared across the order Spirostreptida (Barnett 1997). In all three families, Spirostreptidae, Harpagophoridae and Odontopygidae, the positioning and sorting of minute quanta of sperm, by males and females respectively, determine which individuals have control over fertilization. Finding the different processes responsible for the fertilization success of individuals means answering many questions on both proximate and ultimate levels. In millipedes there is a general trend for instantaneous insemination accompanied by sperm displacement and mixing by males during an adaptive mate guarding phase (Barnett & Telford 1994). This is coupled with female mediation of ejaculate retention after copulation (Barnett *et al.* 1995). The products, patterns and processes of sexual selection in savannah millipedes (Telford & Dangerfield 1996) and the aforementioned studies have provided numerous questions and will form the groundplan

for this parallel study on the forest millipedes of the genus *Centrobolus* Cook, 1897 (Diplopoda: Spirobolida: Pachybolidae).

Millipedes and the novelties of model organisms

Firstly, for the non-specialist, myriapodology is the study of myriapods, which is a loose term uniting the many legged invertebrates of which there are four classes. Millipedes belong to the class Diplopoda and the other three are the Chilopoda, colloquially known as the centipedes, the Pauropoda and Symphyla which are both so little known that they have no common names. Knowledge is fast accumulating largely through the efforts of the Centre de Myriapodologie (CIM) that holds a conference every three years (reviewed in *Acta myriapodologica*, Geoffroy *et al.* 1996). The evolutionary or phylogenetic relationships between the diplopods, chilopods, pauropods and symphylans are mostly unresolved. There are, however, certain taxonomic levels (see Appendix 1 for a complete taxonomy) that I selected because they are based upon reproductive structures or characters. The behaviour of individuals can be observed and scientifically tested to produce a knowledge of how these structures function. The five most important novel biological structures or characters that can be recognised are, in descending taxonomic status, unique to the Diplopoda, Juliformia, Spirobolida and *Centrobolus*.

(i) **Diplopoda** are split into the two subclasses Pencillata (bristly millipedes) and Chilognatha. In all adult male millipedes of the latter group, except those of the order Glomerida (pill millipedes), one or both of the pairs of limbs on the seventh diplosegment are modified into gonopods. Just as the dragonfly males must first load sperm into an intromittent organ evolved on their second and third abdominal sternites (Corbett 1962), so too must millipede males load sperm into the gonopods on their seventh diplosegment.

(ii) **Helminthomorpha** are the “worm-like” millipedes that have an elongate body.

(iii) **Juliformia** are the superorder of helminthomorphs that have a cylindrical body plan. Body volumes can be calculated as $l \cdot \pi r^2$ where l is the length of the millipede and r the radius. Thus body size is perceived as volume which is useful in testing for allometric relationships.

(iv) **Spirobolida**, together with the Julida are the only orders that have both pairs of legs on the seventh diplosegment of the male modified into accessory genitalia, unlike the closely related Spirostreptidae and Harpagophoridae where only the first pair are modified into gonopods and the Odontopygidae where only the sternite of the second remains.

(v) **Centrobolus** species are unique in that they possess tarsal pads, extensions of the last segment of the walking legs (Attems 1928). Tarsal pads have been considered as a taxonomic character but never examined as a secondary sexual character in males.

There are a number of advantages to undertaking a scientific investigation into the mating dynamics of millipedes, and particularly the genus *Centrobolus*:

(i) The speciose nature of the genus makes them representatives of biodiversity. The species diversity of *Centrobolus* is reflected in numerous taxonomic renditions of southern Africa's Spirobolida (Cook 1897, Attems 1928, 1934, Jeekel 1956, Schubart 1966, Lawrence 1967, Hoffman 1971). *Centrobolus* Cook 1897 (Pachybolidae) was resurrected to replace *Chersastus* Attems 1926 (Trigoniulidae) by Hoffman (1971). Thirty-nine nominal species are recognised although the taxonomic status of the genus remains *in limbo* (Appendix 2).

(ii) The male accessory genitalia (gonopods) are complex and have been selected by all taxonomists as their primary characters for identifying species. This facilitates an investigation into genital complexities and their functional roles in sperm competition and female choice (Eberhard 1985). Because the gonopod structures of the two orders

Spirobolida and Spirostreptida have evolved somewhat independently, but have also been derived from a common juliform millipede ancestor, they provide a good comparison.

(iii) Populations can be sampled in large numbers. As with many other southern African millipedes, surface activity peaks in the summer months following the rains (Lawrence 1952, Dangerfield & Telford 1993) and some species emerge in what can be termed a 'swarm' (Sivinski & Peterssen 1997). Highly vagile swarms of *Centrobolus* are almost 100% pure in the sense that they are composed entirely of reproductive stages. A low frequency (<5%) of intercalary males (*Schaltzmännchen*), the stage after an adult male moults and the gonopods regress to a non-functional form, up to now unknown in the Spirobolida, were found in *C. inscriptus* (present study).

(iv) The predictable swarming behaviour at the onset of the rainy season facilitates both field and laboratory based investigations from single populations. *Centrobolus* is confined to the forest habitats (Acocks 1975) on the eastern half of southern Africa, with a sequence of species distributed along a coastal curve passing from east to west and thence to the north as far as Inhambane and Beira in Mozambique (Lawrence 1952, Lawrence 1967: 611). Animals were collected from confined, localized populations without fuzzy boundaries between populations to ensure that the organisms are genetically distinct and of the same species, so avoiding "area-effects".

(v) Individuals can be marked and easily identified with no apparent effect on mating behaviours. Females are willing to mate in quick succession and have sperm storage organs that are easily dissected. The mating history of individuals can be tracked by marking them with unique colour combinations of correcting fluid on the posterior diplosegments. Copula pairs form readily when males are provided with females and can be examined at close range without disturbance.

(vi) These millipedes have determinate growth. A maximum number of segments is

reached and moulting ceases when the adult stage is reached (hemianamorphic) (Hopkin & Read 1992). Thus accurate measurements can be obtained for parameters of body size.

(vii) Finally, what makes *Centrobolus* unusual is that unlike other diplopods, most species of the genus are aposematically coloured and are not permanently cryptic in habit, often being found climbing on trees and shrubs within the forest (Lawrence 1952).

Current controversy

Historically, aspects of copulatory and postcopulatory events involve a confluence of interests (Parker 1984). This school of thought suggests that females may gain from reduced harassment while the male protects paternity. For example, an explanation for the high variability and complexity in species-specific genitalia centres on the function of the male genitalia as internal 'titillation' devices that have been exposed to runaway sexual selection, otherwise known as cryptic female choice (Eberhard 1997). A conflict of sexual interests in the evolution of insect and arachnid mating systems is the latest challenge against widespread cryptic female choice (Choe & Crespi 1997). The so-called alternative to the abovementioned example is that complexity in genitalic form is the consequence of an evolutionary arms-race between males and females and hence the conflict of sexual interests (Alexander *et al.* 1997). Several hypotheses centred around a conflict of sexual interests illuminate weaknesses inherent in widespread cryptic female choice. The male-female conflict hypothesis would predicts that male and female genitalic complexity have interspecific correlations and that there is a non-graded threshold mate choice rather than open-ended preferences. Perhaps the contents of this thesis will resolve some controversy and simultaneously open a gateway for future discussion.

Thesis structure

The chapters are chronologically ordered, beginning with sexual dimorphism in a cylindrical bauplan. This is the starting point for presenting the types of functional ideas that adaptationists seek, e.g. why is one sex a certain size larger than the other, or, why do males possess structures that females lack. Chapters 3 and 4 are morphologically grounded and take identical approaches to figure the anatomy of male and female genitalia. Chapter 5 draws on this morphological evidence by examining the genital biomechanics. The behavioural descriptions in chapter 6, accompanied with quantitative patterns, are used to hypothesize about sperm competition, cryptic female choice and male-female conflicts. Chapter 7 consists of empirical tests based on *a priori* predictions about ejaculate distribution patterns and the dynamics of multiple mating. Finally, chapter 8 gives a short description on the mating ecology of two sympatric species.

Sexual dimorphism in a cylindrical bauplan: a test of Rensch's rule

Historically, explanations for the evolution of sexual dimorphism include niche dimorphism (intersexual competition Selander 1972, Alexander *et al.* 1979, Rising 1987), loading constraints associated with reproductive behaviour (Wheeler & Greenwood 1983, O'Neill 1985, Adams & Greenwood 1987), and morphological constraints associated with internal embryonic development (Price 1984, Greenwood & Wheeler 1985). Potentially non-adaptive influences include genetic correlation between the sexes (Lande 1980), body-size scaling/allometry (Peters 1983, Calder 1984) and phylogenetic constraints or evolutionary inertia (Gould & Lewontin 1979, Ridley 1983, Stearns 1983, 1984, Felsenstein 1985).

Recently, the selective or functional hypotheses for the evolution of sexual dimorphism have been reviewed making them specifically applicable to differences in body size (Fairbairn 1997). The usefulness of finding interspecific trends in sexual size dimorphism (SSD) is questionable. It is debatable whether interspecific allometric scaling is the most pervasive theme underlying all biodiversity (West *et al.* 1997) or simply an epiphenomenon of compiling intraspecific relationships (Kozłowski & Weiner 1997). If the differences in size between males and females are correlated across taxa, it is possible that SSD may reflect different functions depending on relative size of the same body form or "*bauplan*".

Millipedes are severely underrepresented in the allometric analyses of SSD in the phylum Invertebrata. Besides their gonads and accessory genitalia, common sexual differences are known to occur in body mass, length, width and leg dimensions of over half the taxa studied (Telford & Dangerfield 1990a; Hopkin & Read 1992). Unlike the majority of sexually dimorphic species where males are the larger sex, diplopods resemble the majority of invertebrates where SSD is reversed (Thornhill & Alcock 1983). Consequently, larger female body size typically correlates with fitness. The tremendous variation in the selective pressures acting on males and females is common where the males are larger than the females (Darwin 1871, Arak 1988, Shine 1988, 1989, Slatkin 1989, Elgar 1992, Andersson 1994). But when this intraspecific variation in reversed SSD persists, a plethora of other forces may be responsible for its selection.

This chapter investigates sexual dimorphism in the genus *Centrobolus* at both intraspecific and interspecific levels by testing for (1) differences between the sexes that qualify as 'dimorphism'. Dimorphism can be either discontinuous or continuous (Burley 1981). When sexual dimorphism is discontinuous all adult conspecifics can be characterized by sex. When continuous, several possibilities exist based upon the overlap and the area of "ambiguity" where certain individuals could belong to either sex; (2) possible selection pressures that explain these dimorphisms. Selection pressures on body size can be directional, stabilizing, or disruptive (Ridley 1993). Directional selection is for an evolutionary increase or decrease in body size depending on whether individuals of larger or smaller extremes have higher fitness. Stabilizing selection is for the majority of the population to have intermediate body sizes so that selection acts against a change in form. Disruptive selection happens when both extremes are favoured over the intermediate types. Sexual dimorphism is thought to be a common example of disruptive selection; (3)

Rensch's rule predicts that SSD will be negatively correlated with mean body size (hypoallometry) when females are the larger sex (Rensch 1960, Wheeler & Greenwood 1983); (4) discontinuous dimorphism of a secondary sexual character found on the legs of males, the tarsal pads (Lawrence 1967); and finally (5) a discussion that entails prioritising functional hypotheses for the evolution of SSD (*sensu* Fairbairn 1997).

Materials and methods

Morphometrics

Millipedes have symtotic or determinate growth (Hopkin & Read 1992) which means that adult body sizes are fixed and can be measured with minimum error. Males and females were measured in the same manner using the same scale so that both measurement and intrinsic errors were alike for both sexes. Body length (mm) and width (mm) were measured with vernier scale calipers, mass (accurate to 0.01 g) taken by placing animals directly on a Mettler balance, for all the males and females of four species of *Centrobolus*. Basic descriptive figures and distribution plots of the three factors were statistically compared (Mann-Whitney *U*-test; Z-values and Z-adjusted values for normal approximation). Body length : width ratios were also compared (Mann-Whitney *U*-test on arcsine transformed data). The mean values of body length (mm), width (mm) and the number of segments was extracted from published data on 18 species of *Centrobolus* (Schubart 1966, Lawrence 1967). Again comparisons were made between males and females (Wilcoxon matched pairs test).

Allometry

If the size of a juliform millipede is perceived as body volume then it is simply calculated using the formula for a cylindrical bauplan: $l \cdot \pi \cdot r^2$; where l is the length and r the radius of the cylinder. SSD was estimated using the index of $([\text{mean female volume}]/[\text{mean male$

volume]) which can be converted into the sexual dimorphism index of Lovich & Gibbons (1992), $SDI = ([\text{mean female volume}]/[\text{mean male volume}]) - 1$, if a comparison with non-diplopod taxa having male-biased SSD is desired. Allometry for SSD was based on the general allometric model, $\text{male size} = \alpha (\text{female size})^\beta$ (Leuttenegger 1978, LaBarbera 1989). SSD was estimated from log/log plots of the males vs female size. Neither sex was selected as the independent variable *a priori*. The allometric coefficient (β) and the correlation coefficient (r) for log (body size) were measured between the sexes.

Tarsal pads

The ultrastructures of the male walking legs were examined to determine whether they may be modified other than for the support of the body column. A single leg was removed from a midbody ring of dead males for three of the species and viewed using scanning electron microscopy (SEM). Specimens were fixed, first in 2.5% glutaraldehyde (pH 7.4 phosphate-buffered saline) at 4°C for 24 hours, then in osmium tetroxide (2%). Dehydration through a graded alcohol series (50% through 100% ethanol) and critical point drying followed. Specimens were mounted on stubs and sputter-coated with gold-palladium. Tarsal pads were viewed under a Cambridge S200 SEM. The SEMs provided an opportunity to calculate the exact extent of the tarsal pad relative to the tarsal claw. Past taxonomic monographs were also reviewed in order to determine whether this character is species-specific and a potential indicator of sexual dimorphism across the genus.

Results

Intraspecific trends

In 14 of 16 measures female were more variable than males in mass, length, width and volume. (Table 2.1, $n = 4$ spp.). In the exceptions, the null hypotheses that the variances of

the two sexes are equal, were submitted to a variance ratio test (Zar 1984: 123) for which F was calculated as $(SD_{\text{male}})^2 / (SD_{\text{female}})^2$. The exceptions were (i) *C. inscriptus*: $F = 0.32 / 0.08 = 4 > F_{0.05(2) 87, 87} = 1.54$, therefore reject the null hypothesis. The genuinely lower variance in female body mass may be the result of the majority of individuals suffering less from condition-dependence and there being little variance in body mass; (ii) *C. fulgidus*: $F = 50176 / 38809 = 1.29 < F_{0.05(2) 10, 10} = 3.72$. There is no significant difference in the variance of body volumes between the sexes.

Table 2.1 The degree of sexual dimorphism in four millipedes of the genus *Centrobolus* given as the mean $\pm$ SD and coefficient of variation (CV). Significance levels: * $p < 0.01$, ** $p < 0.001$, *** $p < 0.0001$.

	Male	CV	Female	CV	Mann-Whitney U-test	<i>n</i>
<i>Centrobolus inscriptus</i>	2.48 $\pm$ 0.57	23.0	2.27 $\pm$ 0.28	12.3	-6.81***	88
Mass (g)	67.4 $\pm$ 2.9	4.0	63.0 $\pm$ 3.6	5.7	7.13***	88
Length (mm)	5.9 $\pm$ 0.2	3.4	6.7 $\pm$ 0.3	4.5	-11.11***	88
Width (mm)	11.50		9.06		-11.19***	88
Length:width	1841 $\pm$ 175	9.5	2245 $\pm$ 226	10.1	-9.8***	88
Volume (mm ³)						
<i>C. fulgidus</i>						
Mass (g)	1.29 $\pm$ 0.14	10.9	1.97 $\pm$ 0.42	21.3	-3.84**	11
Length (mm)	56.2 $\pm$ 2.5	4.4	63.5 $\pm$ 5.2	8.2	1.23	11
Width (mm)	5.4 $\pm$ 0.2	3.7	6.2 $\pm$ 0.4	6.5	-3.97***	11
Length:width	10.87		9.29		-3.97***	11
Volume (mm ³)	2000 $\pm$ 224	11.2	2501 $\pm$ 197	7.8	-4.57***	11
<i>C. ruber</i>						
Mass (g)	1.28 $\pm$ 0.12	9.4	2.00 $\pm$ 0.48	24.0	4.68***	18
Length (mm)	57.8 $\pm$ 2.6	4.5	62.3 $\pm$ 6.3	10.1	1.34***	18
Width (mm)	5.0 $\pm$ 0.2	4.0	6.1 $\pm$ 0.4	6.6	5.13***	18
Length:width	11.46		10.18		4.07***	18
Volume (mm ³)	1141 $\pm$ 104	9.1	1850 $\pm$ 421	22.8	4.79***	18
<i>C. digrammus</i>						
Mass (g)	0.68 $\pm$ 0.05	7.4	1.02 $\pm$ 0.23	22.5	-2.44	6
Length (mm)	49.9 $\pm$ 2.1	4.2	54.5 $\pm$ 5.4	9.9	-2.29	6
Width (mm)	4.0 $\pm$ 0.1	2.5	4.8 $\pm$ 0.3	6.3	-3.32**	6
Length:width	12.53		11.43		-2.90*	6
Volume (mm ³)	616 $\pm$ 53	8.6	985 $\pm$ 208	21.1	-3.28*	6

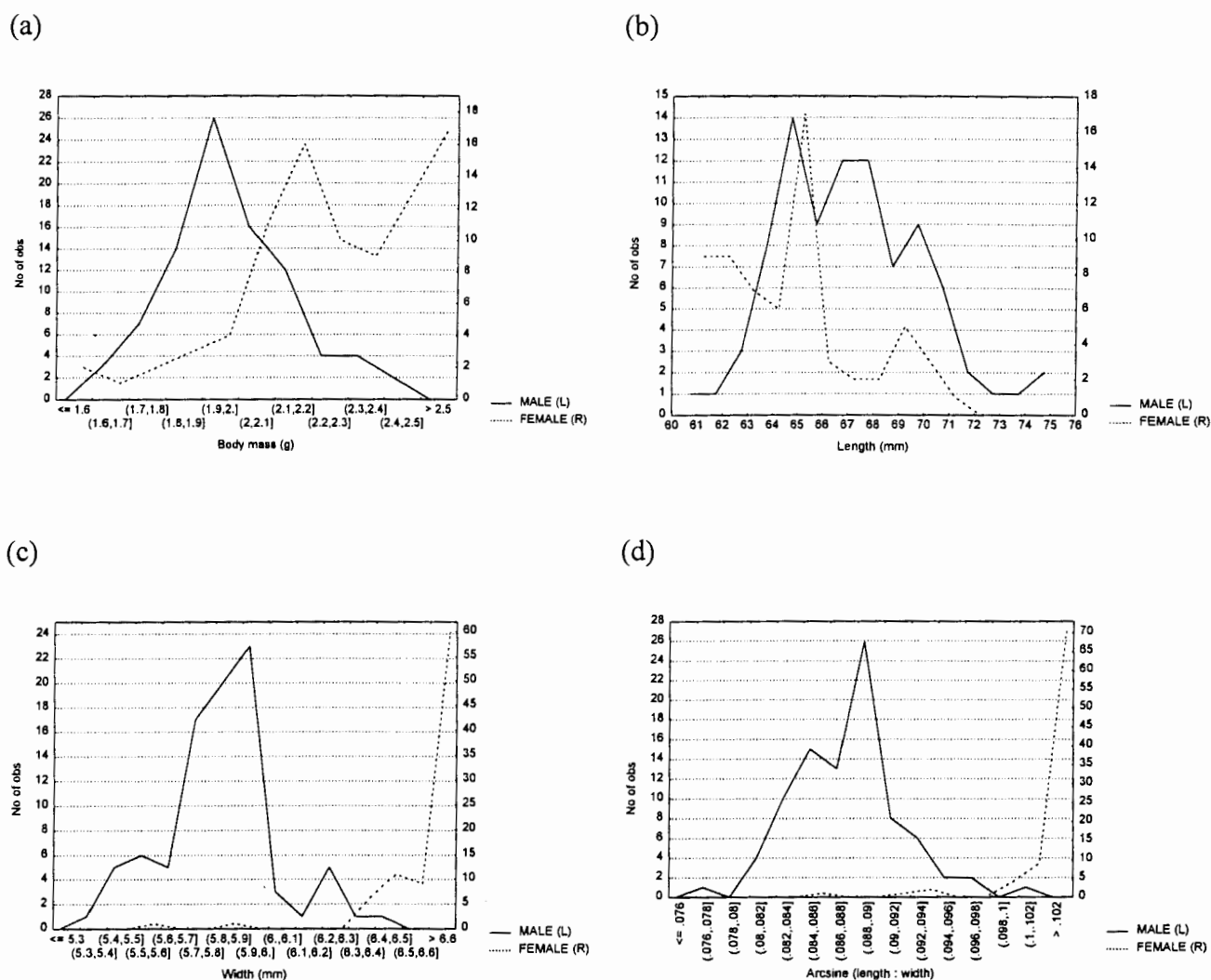


Figure 2.1 The sexual differences in the distribution of mass, length and width (a-c), and the ratio calculated between the latter two (d), for the pachybolid millipede *Centrobolus inscriptus* from a population sampled during the mating season.

Although there is continuous dimorphism in mass, males are normally distributed and females are skewed towards having a larger mass (Figure 2.1a). The double peak may represent a population disruption and directional selection for heavier females. Length is also continuous however the female distribution is skewed towards shorter individuals (Figure 2.1b). Width shows nearly total discontinuity and is strongly skewed towards wider females and intermediate males (Figure 2.1c). The arcsine of length : width ratios places the sexes as equally apart as the distribution for width (Figure 2.1d). The combination of

these two parameters is perceived as sexual size dimorphism as represented by the distribution of body volumes (Figure 2.2).

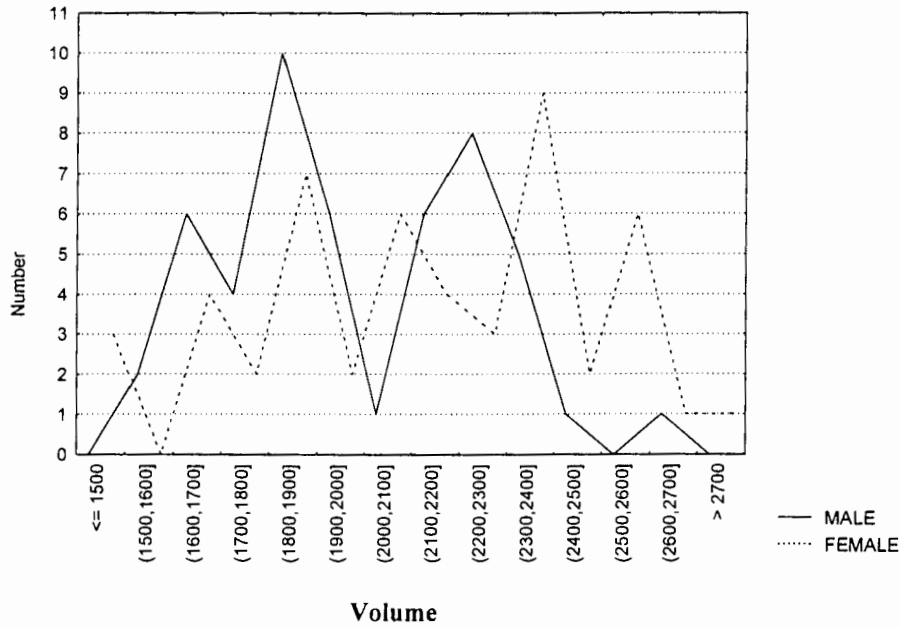


Figure 2.2 The sexual differences in the distribution of body volumes (mm³) calculated for the pachybolid millipede *Centrobolus inscriptus* from a population sampled during the mating season.

Interspecific trends

Centrobolus millipedes range in width from *C. promontorius* at the lower end of the scale up to *C. vastus* (Table 2.2). Generally, females have significantly greater widths (Wilcoxon: $T = 5.00$, $Z = 3.51$, $n = 18$, $P = 0.0005$) and more body rings ($T = 2.00$, $Z = 2.60$, $n = 18$, $P = 0.009$) than males, but are no different in length ($T = 73.00$, $Z = 0.54$, $n = 18$, $P = 0.59$). The differences in width were consistent, i.e. females broader than males, in all except the two species *C. promontorius* and *C. decoratus*. Perhaps these measurements were originally taken from lower stadia (subadult) animals that were collected simultaneously with their conspecific adult males and misidentified as adult females? If so,

that explains their deviation from the general trend of reversed sexual dimorphism in this millipede group.

Table 2.2 Male and female morphometric parameters recorded for the spirobolidan millipede genus *Centrobolus*. All values are given as the mean measurements with sample sizes reliant on the original species descriptions of Schubart (1966) and Lawrence (1967).

SPECIES	MALE			FEMALE		
	length (mm)	width (mm)	Body rings	length (mm)	width (mm)	body rings
<i>C. albitarsus</i> ^L	39	4.0	42	50	6.0	42
<i>C. decoratus</i> ^L	43	4.5	42	31	4.2	42
<i>C. digrammus</i> ^S	41	4.0	45	34	4.4	45
<i>C. dubius</i> ^S	52	5.0	45	51	5.9	45
<i>C. fulgidus</i> ^L	54	5.2	43	52	6.8	44
<i>C. immaculatus</i> ^L	49	4.7	39	60	7.0	40
<i>C. inyanganus</i> ^L	40	4.5	39	43	5.2	42
<i>C. lawrencei</i> ^S	43	4.7	43	43	5.9	43
<i>C. lugubris</i> ^L	53	6.2	45	63	8.4	46
<i>C. promontorius</i> ^S	33	3.6	45	27	3.3	45
<i>C. pusillus</i> ^L	39	4.0	38	40	5.7	38
<i>C. rugulosus</i> ^L	49	5.4	42	50	7.5	42
<i>C. sagatinus</i> ^S	49	6.2	45	48	7.0	44
<i>C. silvanus</i> ^S	46	4.4	43	44	4.8	44
<i>C. titanophilus</i> ^S	28	4.1	43	29	4.3	44
<i>C. transvaalicus</i> ^L	39	4.4	42	38	5.0	43
<i>C. tricolor</i> ^L	45	4.5	43	37	5.2	43
<i>C. vastus</i> ^L	65	6.0	43	63	8.2	44

^S - Schubart (1966); ^L - Lawrence (1967)

Sexual dimorphism ratios (female : male) for length ranged from 0.72 - 1.28 (Mean $\pm$ SD = 0.99 ± 0.15) and width ranged from 0.92 - 1.50 (Mean $\pm$ SD = 1.22 ± 0.18); combined as Length : width the range was from 0.67 - 1.0 (Mean $\pm$ SD = 0.82 ± 0.11). The significant difference between the length : width ratios of males and females ($T = 0.00$, $Z = 3.52$, $n = 18$, $P = 0.0004$), in addition to the difference in width but not length, indicates selection for

larger female size but does not exclude selection for male length. The species in which females had a greater number of segments, but were no different to males in length, indicates that they have shorter squat individual segments than males.

Table 2.3 Body length : width (l : w) ratios and mean body volume calculations for male and female pachybolid millipedes of the genus *Centrobolus*. All values given represent the mean measurements and were taken from the original species descriptions of Schubart (1966) and Lawrence (1967).

SPECIES	MALE		FEMALE		RATIO (FEMALE/MALE)	
	l : w	volume (mm ³)	l : w	volume (mm ³)	l : w	volume/SSD
<i>C. albitarsus</i> ^L	9	490	8	1414	0.89	2.89
<i>C. decoratus</i> ^L	9	684	7	429	0.78	0.63
<i>C. digrammus</i> ^S	10	520	7	523	0.7	1.01
<i>C. dubius</i> ^S	10	1030	8	1389	0.8	1.35
<i>C. fulgidus</i> ^L	10	1147	7	1888	0.7	1.65
<i>C. immaculatus</i> ^L	10	850	8	2309	0.8	2.72
<i>C. inyanganus</i> ^L	8	636	8	913	1.0	1.44
<i>C. lawrencei</i> ^S	9	748	7	1176	0.78	1.57
<i>C. lugubris</i> ^L	8	1600	7	3491	0.88	2.18
<i>C. promontorius</i> ^S	9	336	8	231	0.89	0.69
<i>C. pusillus</i> ^L	9	490	7	1021	0.78	2.08
<i>C. rugulosus</i> ^L	9	1122	6	2209	0.67	1.97
<i>C. sagatinus</i> ^S	7	1464	6	1855	0.86	1.27
<i>C. silvanus</i> ^S	10	704	9	793	0.9	1.13
<i>C. titanophilus</i> ^S	6	365	6	421	1.0	1.15
<i>C. transvaalicus</i> ^L	8	593	7	746	0.88	1.26
<i>C. tricolor</i> ^L	10	716	7	786	0.7	1.10
<i>C. vastus</i> ^L	10	1838	7	3327	0.7	1.81

^S Schubart (1966); ^L Lawrence (1967).

The interspecific correlation between male and female lengths ($r^2 = 0.82$, $n = 18$, $P < 0.05$) and widths ($r^2 = 0.85$, $n = 18$, $P < 0.05$), and the correlation between length and width in males ($r^2 = 0.26$, $n = 88$, $P = 0.02$) and females ($r^2 = 0.32$, $n = 88$, $P = 0.00$) was a predictable result of the juliform cylindrical *bauplan*. When sexual differences were

pronounced in a species all adults could be characterized by sex and readily identified dorsally (Figure 2.3).

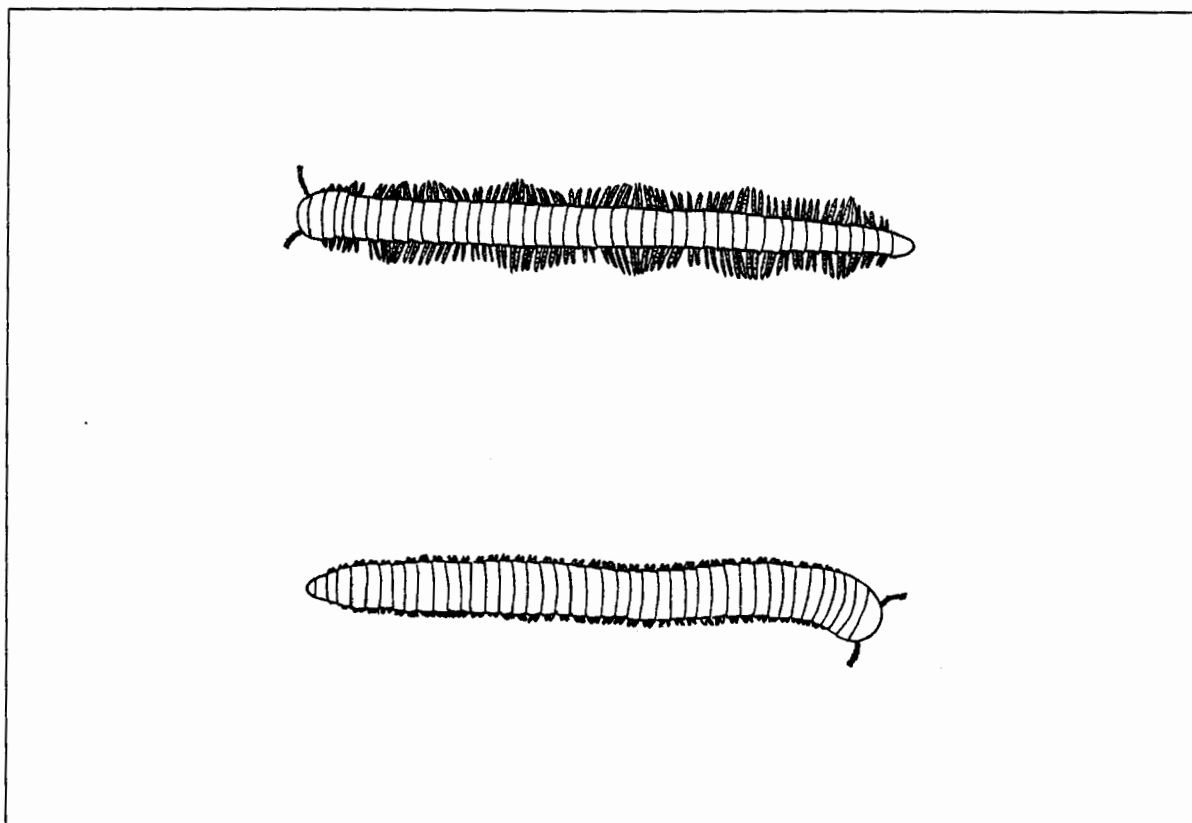


Figure 2.3 Outlined dorsal views of a male (above) and a female (below) pachybolid millipede *Centrobolus inscriptus*, a species showing continuous dimorphism in mass and length but discontinuous dimorphism in width (mean sexual size dimorphism is 1.3 female body volume / male body volume). The legs of the male are visible as a metachronal wave.

The sexual differences between male and female body plans that is observable in many species (Plates 2.1 & 2.2) may not be biologically significant in mating dynamics. The larger variance in the female form, together with the distributions of the measurements taken for *C. inscriptus*, illustrate that most dimorphism is continuous (Figure 2.1).

Allometry

Mean volume ratios in *Centrobolus* ranged from 0.63 - 2.72 (Mean $\pm$ SD = 1.49 ± 0.68) suggesting a relatively high variance of sexual dimorphism within this genus. The regression of male volume on female volume (both log-transformed) is highly significant (Figure 2.4: $R = 0.85$, $F = 42.97$, $df = 17$, $P < 0.0001$) with a positive slope significantly less than 1 ($t = -5.21$, d.f. = 16, $p < 0.0005$), indicating that size ratio increases with body size. Thus, volume ratios indicate that size dimorphism is hyperallometric as predicted by Rensch's rule.

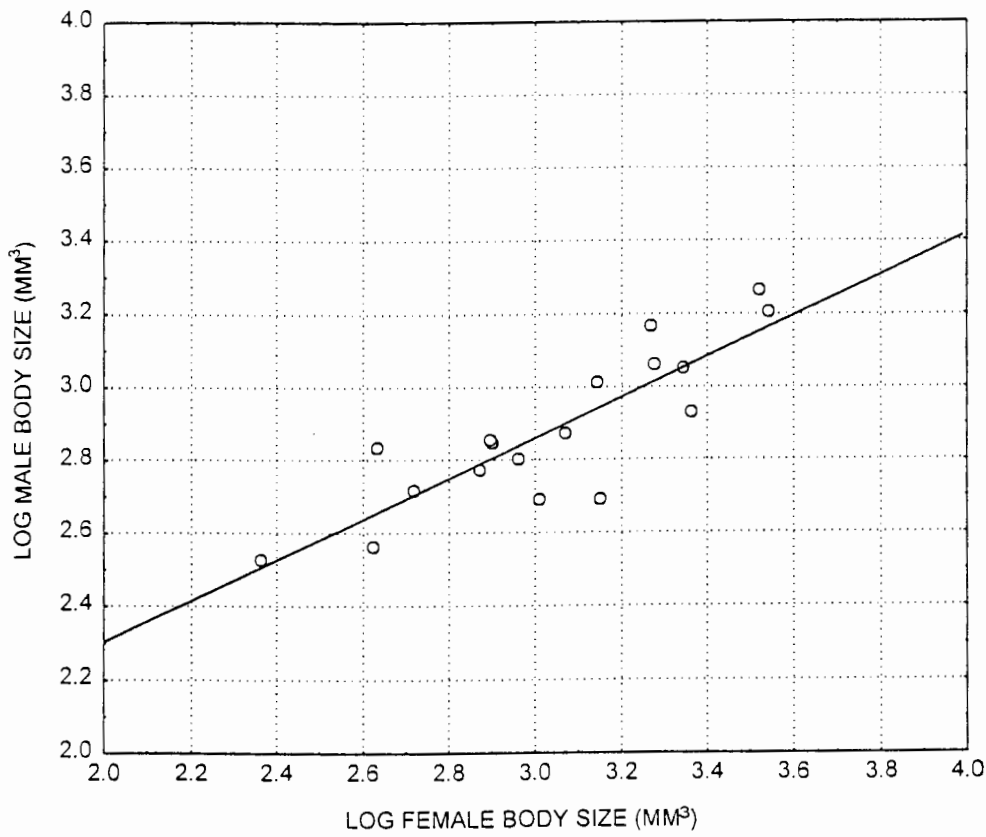


Figure 2.4 Quantitative resolution of Rensch's rule for 18 species of spirobolidan millipedes of the genus *Centrobolus*. Allometry for sexual size dimorphism (SSD) is based on the general allometric model, male size = α (female size) ^{β} (Leuttenegger 1978, LaBarbera 1989). Allometric coefficient, $\beta < 1$ (hypoallometric); correlation coefficient, $r = 0.85$. The regression of log (female size) on log (male size) would generate an identical relationship with $\beta < 1$.

Tarsal pads

Lawrence (1967) used the tarsal pads as secondary taxonomic character to describe eight new species of *Centrobolus*. Differences were noted in their distribution as well as extension relative to the tarsal claw. In my investigation, the tarsal pads of *C. inscriptus* are robust and prominent ($\pm 1018\mu\text{m}$, $n = 10$), extending to about the midlength of the tarsal claws. In comparison, the tarsal pads of *C. anulatus* ($\pm 540\mu\text{m}$, $n = 8$) are relatively abbreviated. The ratios of tarsal pad : total tarsus were calculated from the SEMicrographs as 1.04 and 0.83 for the two species respectively¹. *C. digrammus* exemplifies how each pad is subtended on the oral and aboral sides by setae and a gripping ridge around the periphery (Plate 2.3C).

¹ A quantitative morphological comparison of this type is useful when examining the mating dynamics of two sympatric species as can be found in chapter 8 of this thesis.



Plate 2.1 Sexual dimorphism in spirobolidan millipedes: A. *Centrobolus fulgidus*, both sexes are conglobated (coiled up) showing the larger female; B. *C. ruber*, the male is the brighter with longer legs; C. *C. digrammus* male, displaying the legs with the elaborate tarsal pads; D. *C. digrammus* female, legs are smaller and cannot be seen from the dorsal aspect.

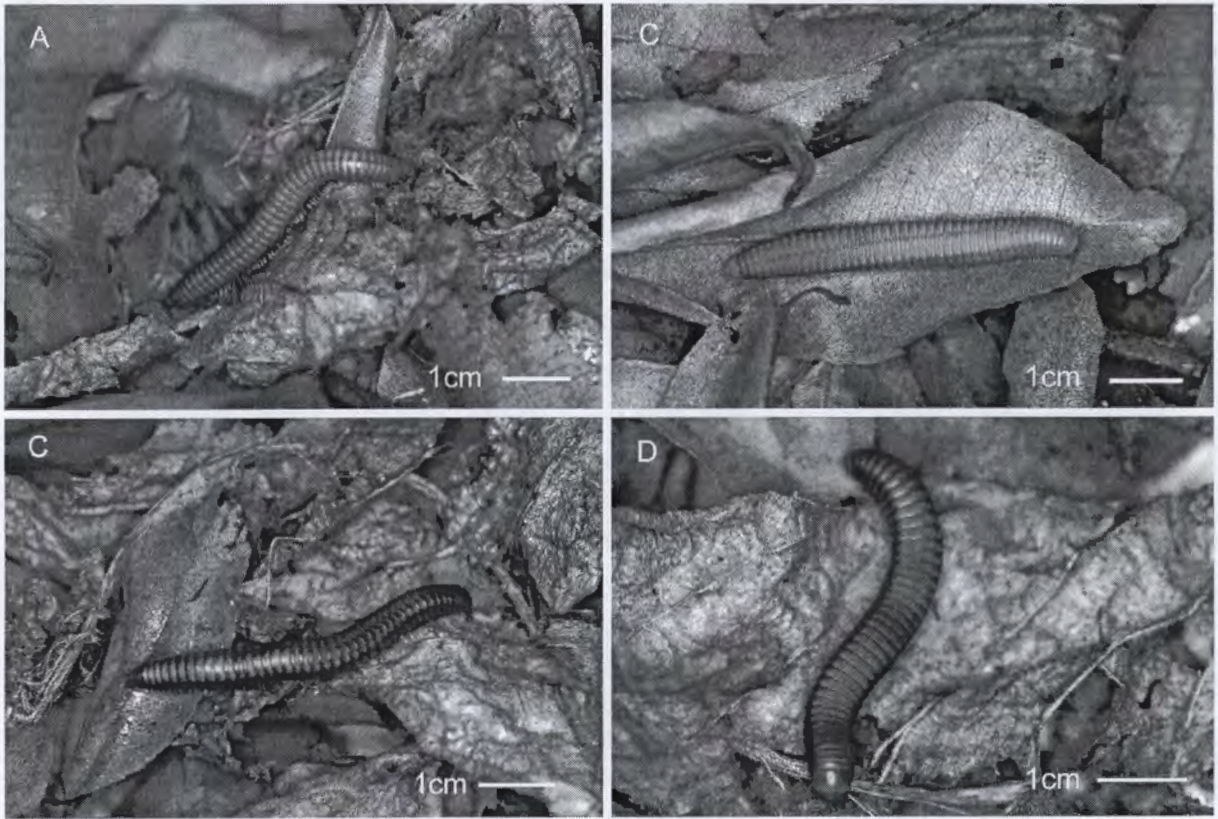


Plate 2.2 Sexual dimorphism in spirobolidan millipedes: A. *Centrobolus striolatus*, surface active male displaying how the legs equipped with tarsal pads extend beyond the cylindrical body; B. *C. striolatus*, female with the short squat cylindrical bauplan; C. *C. strigosus*, surface active male; D. *C. strigosus* female feeding amongst leaf litter.

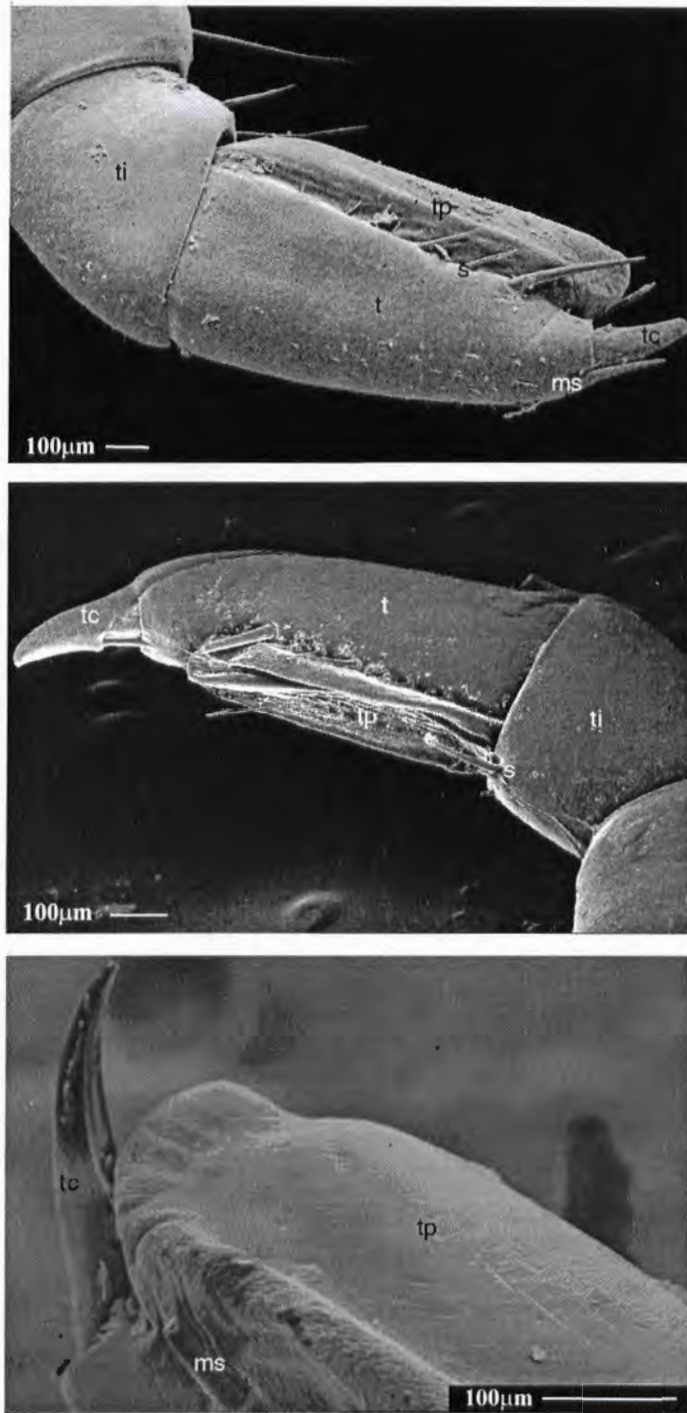


Plate 2.3 Distal podomeres of typical male walking legs from the midbody segments showing the tarsal pads (tp): A. *Centrobolus inscriptus*; B. *C. anulatus*; C. *C. digrammus*. macroseta (ms), postfemur (pf), seta (s), tarsus (t), tarsal pad (tp), tibia (ti), tarsal claw (tc).

Discussion

Sexual dimorphism is accepted to reflect different selection pressures operating on males and females. In animals like millipedes where both males and females mate multiply (polygynandry), SSD becomes difficult to explain since there may be sexual selection pressures for large size in both males and females. SSD is further obscured by natural selection when fecundity is related to female size. Furthermore, because SSD is the ratio of two quantities, either may have changed over evolutionary time. Eight functional hypotheses have recently been suggested for the evolution of allometry for SSD (Fairbairn 1997). Certain of these hypotheses are more applicable for generating predictions about the interspecific allometry ($\beta=1.3$) for SSD and intraspecific patterns in *Centrobolus*.

Elongating males or expanding females?

The reasons for the pattern of reversed SSD in millipedes can be debated in the same way that arachnologists are considering the evolution of sexual dimorphism in spiders (Coddington *et al.* 1997). Just as spider sexual dimorphism may be the product of female 'gigantism' or male 'dwarfism', or both, so too may millipede sexual dimorphism be the product of male 'elongation' and female 'expansion'. There is still debate over whether the ancestors of millipedes had long bodies which reduced in number of segments during evolution (contraction theory), or whether they became gradually longer (elongation theory). As an exponent of elongation (Verhoeff 1926-1932 cited in Hopkin & Read 1992), expansion must also be considered because of the non-linear increase in length with girth when maximizing the volume of the cylindrical bauplan.

Large size accelerates the evolution of SSD by facilitating response to sexual selection (Clutton-Brock *et al.* 1977, Gaulin & Sailer 1984). This hypothesis derives from the

assumption that energetic or competitive constraints on increasing size are relaxed in larger animals. Owing to the cylindrical bauplan of the juliform millipedes, maximizing body volumes through a twofold increase in radius can only be matched by a fourfold increase in length. The energetic costs associated with increasing body diameter are probably less than would be associated with increasing body length (Telford & Dangerfield 1996). Also, the females, being larger, can be expected to have lower mass-specific metabolism (see Frears *et al.* 1996 on the metabolism of southern African millipedes). General observation confirms this is also true with *Centrobolus*, males are the more active sex and predictably the more competitive for mates. So female reproductive success can be maximized through an increased body size with relatively low energetic costs compared to male reproductive success.

Variance dimorphism

Because the phenotypic variance for body size differs between the sexes, a given intensity of disruptive selection has produced a greater response in the females, the sex with the larger variance. This has resulted in both reversed SSD and the allometric function (β) of 1.3. Genetic correlation between males and females in body size are known to produce non-adaptive allometry for size dimorphism through a feedback mechanism in which frequency-dependent selection in one sex leads to a correlated response in the other sex and a change in the average size of the species. A good example is the model for non-adaptive evolution of size-ratio allometry based on differences in body size variance (Cheverud *et al.* 1985). Because no allometry is predicted at equilibrium, but remains transitory, this hypothesis is rejected

Assuming that body size affects the reproductive success for both sexes equally, when females are significantly larger than the males, average reproductive success is expected to increase with body size in females more so than in males (Howard 1988). The lower variance in male length suggests that directional selection for longer males leading to higher reproductive success cannot be excluded.

Phylogenetic constraints

A constant degree of sexual dimorphism across all *Centrobolus* species may represent simple inheritance from a common ancestor or several independent origins. Unfortunately a close phylogeny of *Centrobolus* is unavailable so each species represents a separate data point. The presence of obvious sexual dimorphism in most *Centrobolus* millipedes argues for the general pre-eminence of strong intrasexual selection. SSD can be used to index other sexual interactions and eventually sexual conflicts, competition and choice, but not phylogeny. There is no answer as to how the tarsal pads might have evolved during phylogeny but their presence as a discontinuous dimorphic character may be useful in distinguishing the sexes and so provide another approach to determining a cause of sexual dimorphism in *Centrobolus*.

Is fecundity selection for volumetric females stabilizing?

Owing to the differential variance between male and female body form, numerous selection pressures are potentially responsible for the patterns of SSD found in *Centrobolus*. The production of large numbers of eggs by female millipedes requires a large ovary to store the ova prior to clutching. The ovary is situated in the abdominal cavity which consequently has a larger volume than in males. Size generally correlates with the number of eggs contained inside the females (in polydesmoid millipedes, Bhakat *et al.* 1989;

Glomeris marginata Heath *et al.* 1974). If this is also the case in *Centrobolus*, then the maximum width of the female is a determinant of fecundity and female body size increases through fecundity selection; a form of directional selection where larger females benefit from the positive effects on fecundity. Hence if directional selection for increased fecundity could produce non-equilibrium female-biased size dimorphism, then why do males retain the lower variance? Smaller females are known to have higher fecundity in the forest julid millipede *Rossiulus kessleri* (Striganova 1996). Larger volume cannot be assumed to indicate fecundity selection without first examining selection on male length.

Alternative hypotheses

Body size and SSD can covary when both are influenced by the distribution of a resource (Alexander *et al.* 1979, Clutton-Brock *et al.* 1977, Webster 1992). Incorporated into these hypotheses is the proposal that breeding aggregations favour large size in females because larger females are more successful in intrasexual competition (Webster 1992). Female dispersion would have to have a proportionately greater effect on male size than on female size. Other factors, such as sexual differences in palatability, as described for the monarch butterfly (Brower & Glazier 1975), could lead to sexual differences in body proportions.

Clues from a sexually selected character

Allometry consistent with Rensch's rule can be attributed to allometric growth of secondary sexual characters in males (Rensch 1960). Despite female forest millipedes having greater body volumes, their tarsal pads are ambulatory (abbreviate) relative to those of the male. Thus, the elaborate tarsal pads of the males are not an adaptation for supporting the body column, and if they are not under any other form of natural selection (Darwin 1871), their function to grip females before and during copulation, by increasing

the potential reproductive rates of males, they can be expected to correlate with body size. Even cryptic female choice can be predicted from this kind of sexual dimorphism, e.g. in the wings of decorated crickets *Gryllodes sigillatus* (Sakaluk 1986).

Correlational selection

When mating pairs engage in prolonged copulation the mating success of males may depend significantly on their size relative to their mates, i.e. SSD (Adams & Greenwood 1987, Bastos *et al.* 1996, Bourne 1993, DeFrenza *et al.* 1986, Fairbairn 1990, Marden, 1989, Naylor & Adams 1987). Consequently SSD can be expected to correlate with any selective pressure that exists on the cylindrical form. Assuming that the optimum and standard deviation of the function relating body volume to survival rate are identical for males and females (Hedrick & Temeles 1989), then consistent SSD should be proportional to the difference in the reproductive selection gradients between males and females. This provides an alternative to that where sexual selection on males is not expected to change the equilibrium body size of females (Lande 1980, Slatkin 1989).

Although largely continuous dimorphism exists in the body volumes of male and female *Centrobolus*, the possession of a cylindrical bauplan under disruptive selection indicates that divergence between the sexes with reversed SSD is consistent with Rensch's rule. More than one kind of selection pressure is operating in males and females of the same species (Table 2.4) but the 1.3 dimorphism slope indicates a common pressure across the genus. The allometry of sexually selected characters remains untested in millipedes.

Elaborate male genitalia: the shape of cryptic female choice

Millipedes display clear sexual dimorphism in their primary sexual characters (Hopkin & Read 1992). The external openings of the male and female reproductive tracts are anteriorly situated (i.e. millipedes are progoneate), stemming from either a pair of penes on the third diplosegment or a pair of vulvae on the second diplosegment, respectively. The sperm are not deposited directly onto the eggs during copulation but are indirectly transferred from the male penes to the female vulvae (Blower 1985). This is accomplished by a pair of accessory intromittent organs, known as gonopods or “sex-legs”, that are modified legs of the seventh diplosegment. The complex gonopods are species-specific characters that supersede the primary male genitalia in taxonomy (Kaestner 1968, Shear 1976, 1981, Shelley 1981). Consequently, the functional morphology of millipede gonopods has also been examined (Barnett 1991, 1997, Barnett *et al.* 1993, 1995, Barnett & Telford 1996, Tadler 1993, 1996a).

The genital structures of both odonates and diplopods have evolved rapidly and divergently and another commonality is that sperm transfer is indirect (Waage 1979; Miller 1991). These structures are known to have dual functions in transferring sperm and displacing rival's sperm from within the female spermatheca of spirostreptidan millipedes (Barnett *et al.* 1991, Barnett *et al.* 1995, Barnett 1997). Slight structural differences in the gonopods can affect these functions. The gonopods of *Centrobolus*, being of the order Spirobolida, do not develop as a gradual modification of functional walking legs but pass through a progressive growth stage or differentiation of their own (Berns 1968), and both pairs of legs on diplosegment VII are modified (Figure 3.1). In other families, e. g. Spirostreptidae, it is only the front pair of

legs that are modified. Whether these structural differences have evolved because separate structures function in sperm displacement and transfer remains to be tested.

A copulating pair of millipedes *Cylindriulus punctatus* (After Haacker & Fuchs 1970), as featured by (Eberhard 1985), exemplify how all five general hypotheses for the evolution of genitalia are testable in organisms with accessory genitalia such as millipedes. Subsequently both Barnett (1991) and Tadler (1993) adopted millipedes to test the range of hypotheses (reviewed in Eberhard 1985). In capsule form these are: (1) ***Lock-and-key hypothesis*** (Dufour 1844, Mayr 1963): females remain unfertilized by males of other species because their complicated genitalia permit insemination only by the corresponding gonopods of conspecific males. The male has the gonopodal “key” to fit the females “lock”; (2) ***Genitalic recognition hypothesis*** (Stephenson 1930, Hammond 1981): male gonopods function in species-specific ways that stimulate females to trigger a reproductive signal; (3) ***Mechanical conflict of interests*** (Lloyd 1979, Wing 1982): an evolutionary arms-race where males evolve structures to get sperm past female resistance and into preferred sites. Wing (1982) suggested that male genitalia might function to damage female copulatory structures and thus prevent further copulation, and then females would evolve protection against such damage; (4) ***Pleiotropism hypothesis*** (Mayr 1963): a change in the genetic constitution of the species produces an incidental change in the structure of the gonopods, which “are less subject to the corrective influences of natural selection...provided the basic function of gamete transfer is not impaired (Eberhard 1985: 104).”; Finally, a more general hypothesis is that by (5) ***Cryptic female choice*** (Thornhill 1983, Eberhard 1985: 70): “females discriminate among male’s of their own species on the basis of the males genitalia, and that males with favoured genitalic morphologies sire more offspring than others.”

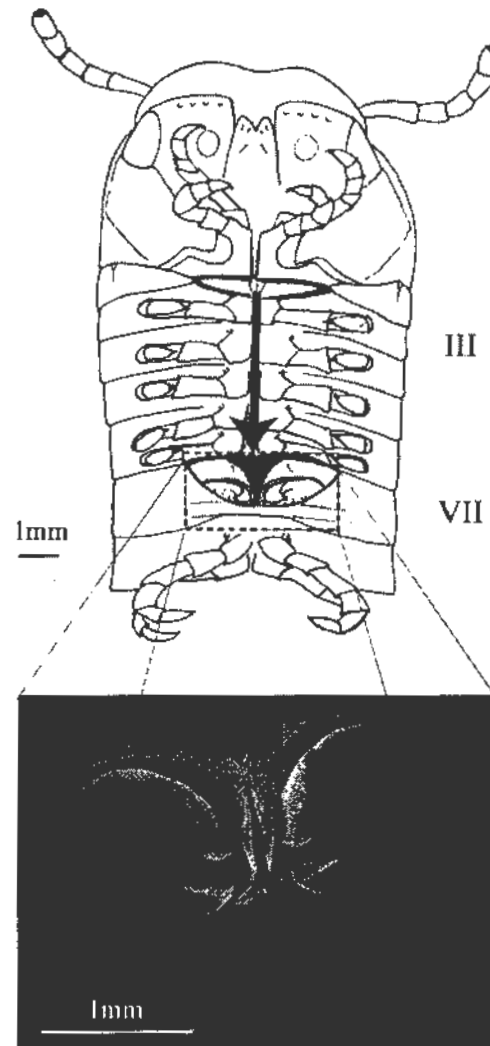


Figure 3.1 Ventral view of the anterior region of a male pachybolid millipede *Centrobolus* (above) showing the reproductive organs that are found near the head (i.e. progoneate). Sperm passes (arrow) from the penes that open at diplosegment III to a pair of gonopods or "sex-legs" present on diplosegment VII. The gonopods have been dissected out and examined under the scanning electron microscope (below) to show complexities besides those necessary for indirect sperm transfer.

Materials & Methods

Four species of *Centrobolus* were delimited *a priori* based on morphological grounds¹. The gonopods were dissected from dead males of the four species and prepared for scanning electron microscopy (SEM). Specimens were fixed, first in 2.5% glutaraldehyde (pH 7.4 phosphate-buffered saline) at 4°C for 24 hours, then in osmium tetroxide (2%). Dehydration through a graded alcohol series (50% through 100% ethanol) and critical point drying followed. Specimens were mounted on stubs and sputter-coated with gold-palladium. Gonopods were viewed under a Cambridge S200 SEM. SEM micrographs were examined and the individual components of the gonopods were identified according to the available species descriptions. Two sets of linear measurements were made from the SEM micrographs: (1) the prominence of the sternite. This has been estimated before as a ratio of how far it extends from the basal region up to the top of the coleopods; (2) the length of the spines present on the distal knob were estimated from measurements taken directly from SEM images.

Results

The gonopods of *Centrobolus* are incredibly complex and do not resemble the walking legs in the slightest. The advantage of the SEM approach is that it revealed all the structures with possible functions in lock-and-keys, mechanical and sensory recognition, conflict of interests and copulatory courtship. Each gonopod consists of an anterior and posterior sex-leg. The phallopods, being the posterior legs, are hidden behind the anterior legs. The coleopods, being the anterior legs, are visible without magnification when males are viewed ventrally (Figure 3.1).

¹ This assured that later circularity was not absolute because the male gonopods are primary taxonomic characters.

The collection of SEM micrographs for each species are particularly more informative when comparisons are made between congruent views. ²Potentially informative regions were identified and labelled on the relaxed gonopods of the following four pre-defined species:

(i) *C. anulatus* (Plate 3.1)

Coleopods: The basal joint (b) is angular on the inner side. The distal joint (d) bears a lappet near the terminal thickening, the latter beset with little conical points that will be termed the "spines". Phallopods: The distal lamellae (dl), more appropriately termed the lappet for obvious and also potentially functional reasons, are broadly rounded and low, not reaching the margin. (cf. Attems 1934, fig. 50).

(ii) *Centrobolus inscriptus* (Plate 3.2)

Coleopods: The sternite (v) is triangular and prominent up to the middle of the gonopod (Table 3.1). The basal joint (b) is broad and rounded at the tip while the second joint (d) is somewhat narrow with two lateral lobes. The summit (k) of the telopodite is covered with little knobs and spines seated in pits. ³Phallopods are not visible from the plate: Lawrence's (1967) description of a broad tibial process (ti) that projects a little. The lappet differs from the previous species in that it extends beyond the margin. (cf. Attems 1928, Plate X figs 231-233).

(iii) *C. fulgidus* (Plate 3.3)

Coleopods: The distal joint (d) has an elongated lower lobe produced laterally plus three ridges medially. The distal region of the coleopods are not as broad in the other three

² The species descriptions given here are congruent with those originally described and accompanied by line drawings made through light microscopes and hand lenses by Attems 1928, 1934, Jeekel 1956, Schubart 1966, Lawrence 1967, Hoffman 1971. The original abbreviations have been retained wherever possible (Appendix II).

³ The position of the phallopods is such that they are seated behind, and protected by the coleopods. The static examination of the gonopod ultrastructures did not reveal the phallopods but the entire structural complexity of the phallopod is revealed from an examination of their mechanics *in copula* depicted by Plates 5.4-5.5.

species, none of which have three lobes. Phallopods: The lappet (dl) near the tip tapers towards a triangular groove on the upper side of the tibia distally (cf. Lawrence 1967, figs 35,36).

(iv) *C. ruber* (Plate 3.4)

Coleopods: The basal joint (b) has a straight internal margin and oblique terminal margin, connected by a wide arch with the outer margin. The tip of the second joint has a thick black knob, bearing a little lobe (Table 3.1; Attems 1928, fig. 221). The lappet of the distal joint (d) is close to the terminal thickening. The sternite is pointed and relatively short (Table 3.1; Attems 1928). Phallopods: The tibial process (ti) is broad, projecting but little, with a smooth margin; between the tibial process and the tip is a pointed lobe (Attems 1928), the distal lappet (dl) is longer than wide. (cf. Attems 1928, Plate IX figs 221-223, Attems 1934, Plate XXV figs 52, 53).

Table 3.1 Male genital measurements of four *Centrobolus* millipedes that were estimated from scanning electron micrographs taken at consistent angles (Plates 3.1-3.4). Sternite prominence was estimated from single specimens and the number of spines present was also counted (*n*)

Species	sternite prominence	spine length	<i>n</i>
	(% of total gonopod height)	(μm)	
<i>C. anulatus</i>	30	5	21
<i>C. inscriptus</i>	50	10	10
<i>C. fulgidus</i>	35	-	-
<i>C. ruber</i>	25	2.5	23

- spines were absent.

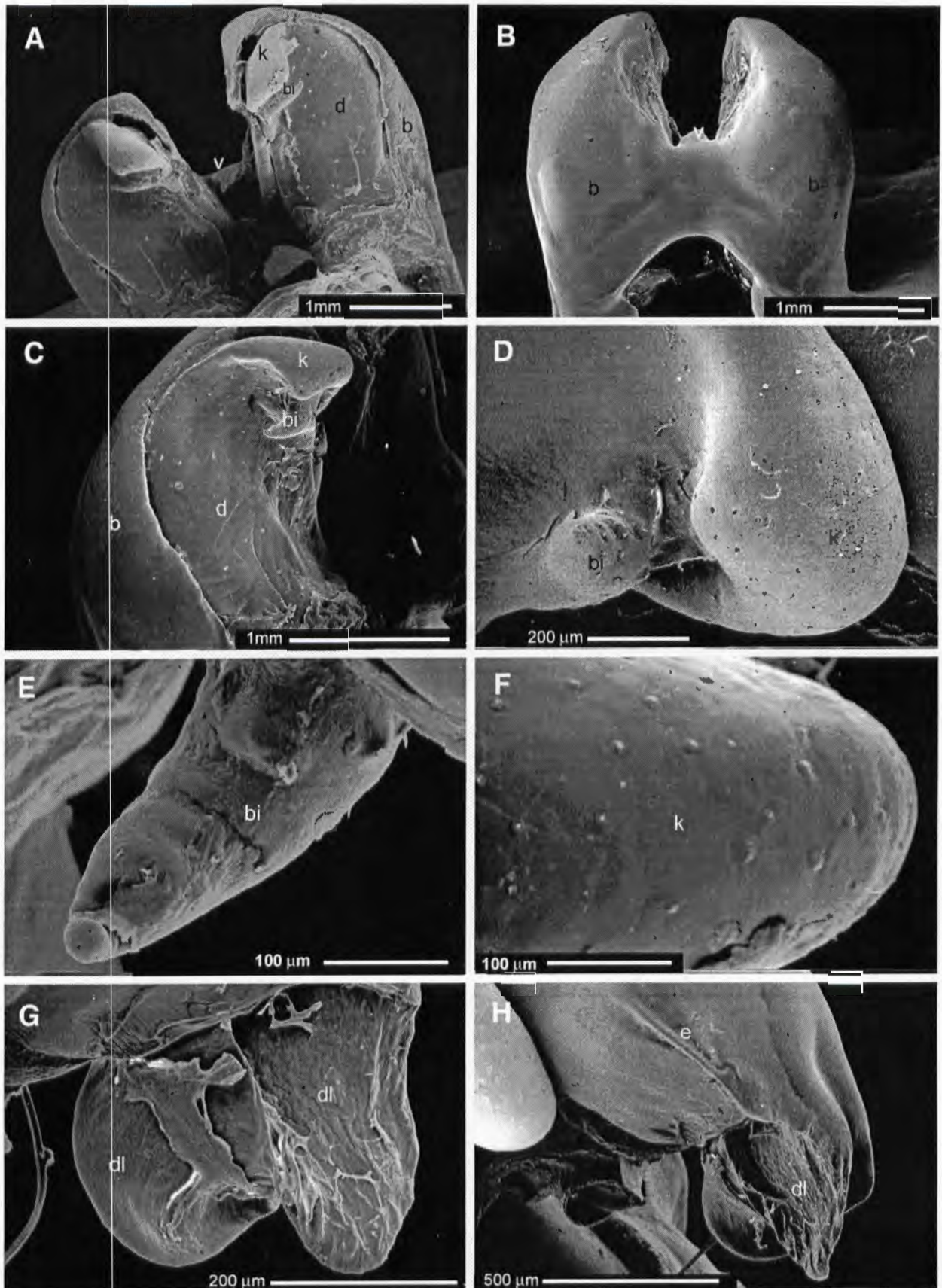


Plate 3.1 *Centrobolus anulatus* gonopods. A. Aboral view of the coleopod showing its distal (d) component carrying the knob (k) and the "bill" (bi) and its basal (b) component; B. Oral view of the coleopods with the sternite (v) positioned medially, C-D. Lateral views of the right distal telopodite lobe, E. Lower distal telopodite lobe or bill, F. Surface of the distal telopodite lobe knob, G-H. Distal lamellae (dl) of the phallopod with a prominent chitinous ridge (e).

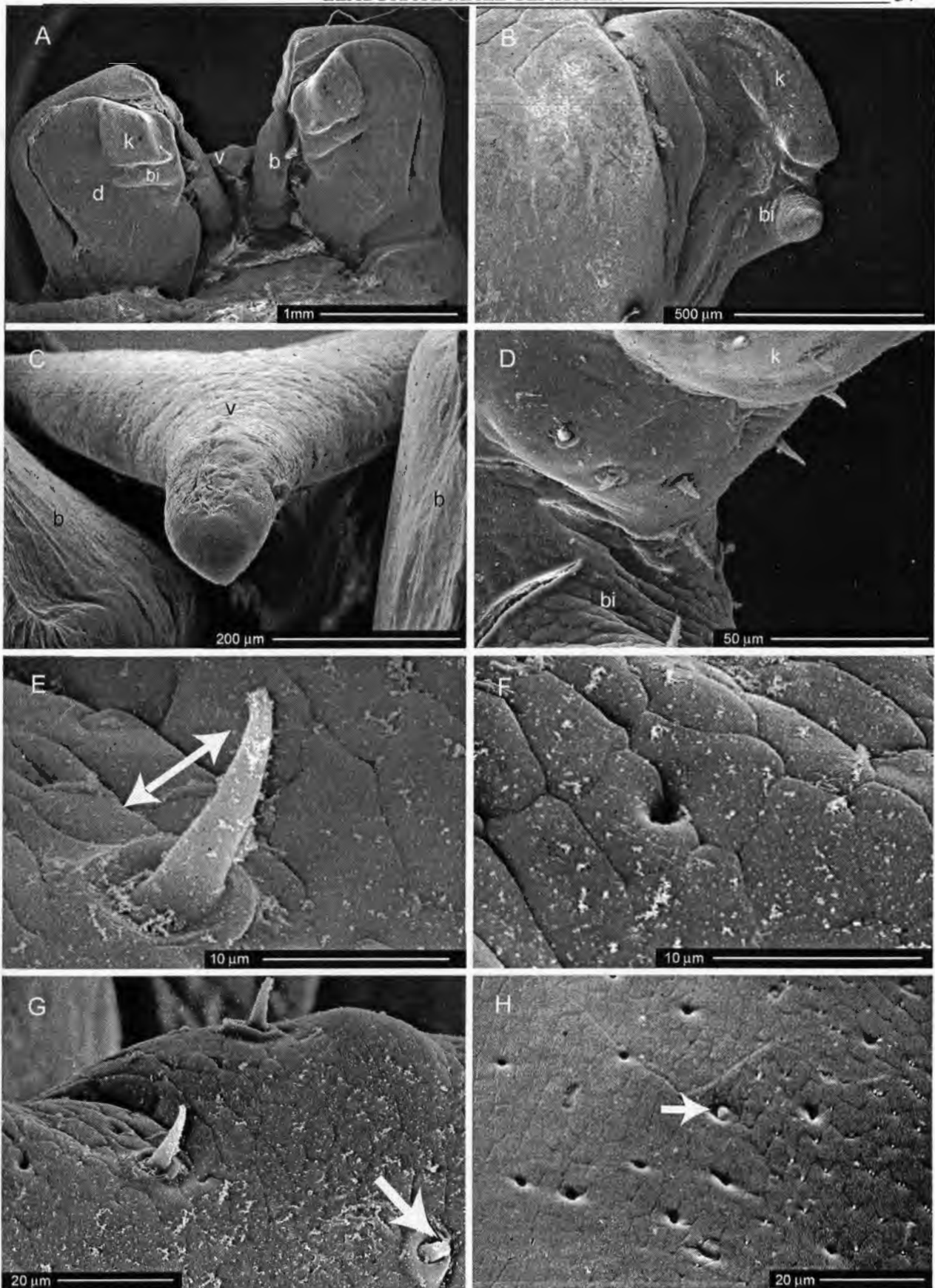


Plate 3.2 *Centrobolus inscriptus* gonopods. A. Aboral view of the coleopods; B. Lateral views of the right distal telopodite lobe bearing the knob (k) and bill (bi); C. Aboral view of the stenite (v) emerging medially between the basal joints (b) of the coleopod; D. Topology of the distal telopodite lobe; E. The speculated sensory spine with the (arrow) indicating its movement against the basal cell; F. A surface pit; G. Closer examination revealed damage to some of the spines (arrow) possibly inflicted *in copula*; H. Surface pit with a trapped particle (arrow).

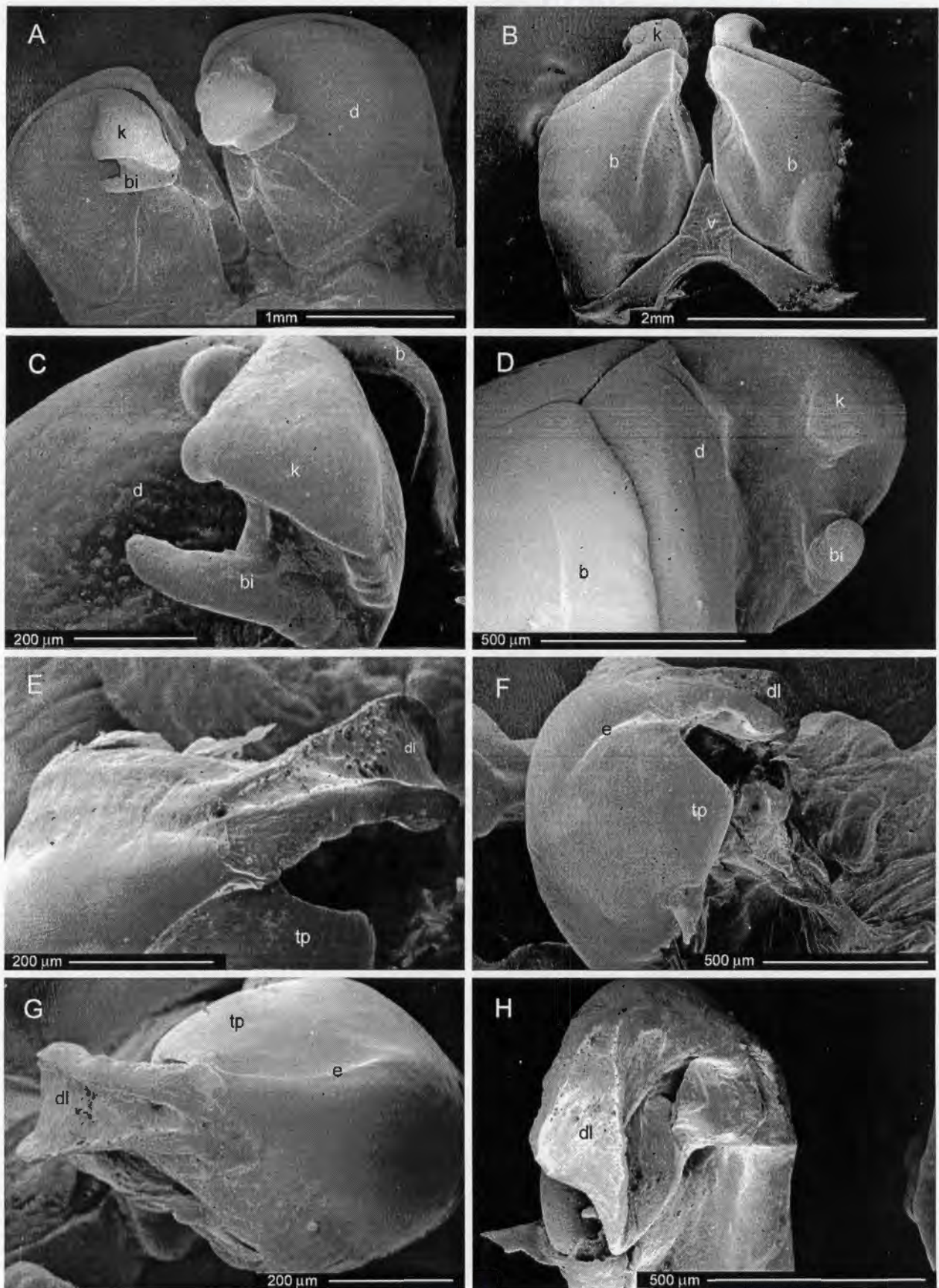


Plate 3.3 *Centrobolus fulgidus* gonopods. A-B. Aboral and oral views of the coleopods showing the distal (d) region with a lobe bearing the knob (k) and bill (bi), the basal (b) component and the sternite (v); C-D. Aboral and lateral views of the distal telopodite lobe showing the slender species-specific bill and knob; E. The recurved distal lamella (dl) of the phallopod with a triangular gap aside the tibial process (tp); F. The tibial process is flanged; G. The chitinous ridge is extensive; H. The "scoop" exemplifies an elaboration of an already complex structure.

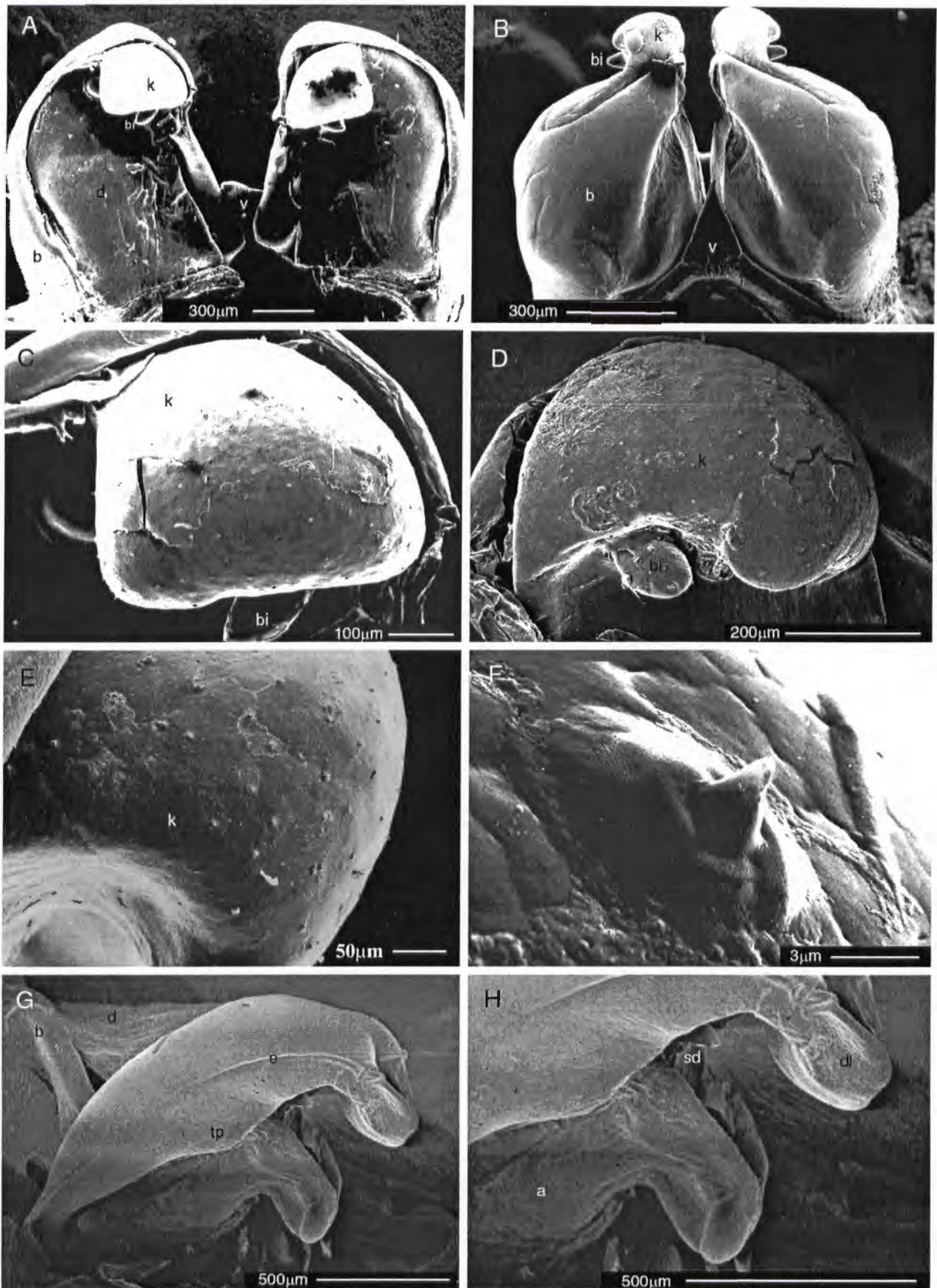


Plate 3.4 *Centrobolus ruber* gonopods. A-B. Aboral and oral views of the coleopods showing the slender distal telopodite region (d) with hidden bill (bi) and rounded knob (k) visible above the basal region in oral view; C-D. Aboral and medio-lateral views of the distal lobe focussing on its reduced components; E. Miniature spines associated with the knob; F. A single spine; G. The tapered phallopod and reduced tibial process (tp); H. The distal lamella has no scoop.

Discussion

The divergent evolution and complexity of gonopods is highly probable in a speciose genus like *Centrobolus* (Appendix 1). Although taxonomic review alone reveals the gonopods as the primary taxonomic characters, this is insufficient evidence for their rapid evolution and divergence. Reasonable deductions can be made concerning each hypothesis for the evolution of complex male genitalia from the static morphologies of *Centrobolus* gonopods simply by examining the component structures to discover their potential functions in copulation.

The indifference of pleiotropism

Pleiotropism has been used to explain complex gonopods in the past (Kraus 1968), however it is no longer considered testable in animals possessing secondary genitalia, as Eberhard (1985) points out: “[T]hat the supposed pleiotropic effects should fail to appear in just these primary genitalia not involved in copulation and should be so consistently present in just those structures used in copulation defies explanation in terms of pleiotropism. The theory supposes that there are only chance associations between allele changes and their pleiotropic effects on genitalia, but the association between rapid and divergent evolution and direct contact with mates is so strong that it discredits the theory.”

Keys without locks?

In *Centrobolus*, the combinations of structures, spines and pits, distal and basal joints, and the double paired gonopods themselves, are all potentially interlocking features. Besides the inherent circularities that the original lock-and-key hypotheses are fraught with (reviewed by Shapiro & Porter 1989), it is neither an alternative to a conflict of interests nor recognition hypotheses. The actual “keying” process of the male genitalia into the female may be

conflicting when the gonopods insert, twist and turn inside the female. Whether there is a recognition system involved or whether the movements performed by the male stimulate the female cannot be determined from male genital morphology alone. The earliest functional explanations for millipede gonopods that were in accord with the original lock-and-key hypothesis (Verhoeff 1926-1932) are outdated. Interspecific mating experiments remain the only way to really falsify a classic lock-and-key hypothesis⁴, however this is not a prerequisite for testing the next three hypotheses.

Conflict of interests

Setae and spines that are associated with the male gonopore in earthworms hold the copulating pair together (Stephenson 1930) as do similar structures found in some insects (Thornhill & Alcock 1983). The damaged spines discovered on the gonopods of *C. inscriptus* suggest mechanical wear. Whether this is the outcome of a conflict of interests or a by-product of recognition or stimulation remains to be tested. Further examination of the structures would be uninformative unless they are costly to produce. This is doubtful given their relative size (to body) and number (to legs). The last alternative is that they perform other functions during copulation such as stimulating female choice. If there is a conflict of interests between the sexes then (1) multiple mating by females; (2) incomplete sperm precedence; and (3) fertilization separate from insemination are obvious predictions.

(Specific mate) recognition

Some females are able to discriminate males on the basis of their grip through tactile stimuli

⁴ Conducted in order to determine whether it is possible for the males of one species (*C. inscriptus*) to effect mechanical coupling of genitalia with a heterospecific (*C. anulatus*) (Chapter 8). This is a phenomenon that has only once been demonstrated in other animals that occur in geographical sympatry (Paulson 1974). Together with consideration as to whether normal amounts of sperm get transferred to regions in the female where they may be used for fertilization, this is the evidence needed to clearly refute a lock-and-key hypothesis for gonopod evolution.

received by the basiconic mechanoreceptors (Robertson & Paterson 1982). I cannot envisage a female process that would have evolved 'for' the female to discriminate among males from different species while simultaneously discriminating individual males. The process of recognition, like the lock-and-key, is unlikely because reproductive "isolation" is more likely to occur during the precopulatory and postcopulatory stages of mating (Eberhard 1996).

Cryptic female choice for spines on the summit

The positive association of spine length with sternite prominence is worthy of note since it could mean directional evolution in gonopod form, and that those forms may be chosen by females. There is no direct evidence for a Fisherian runaway effect in gonopods but it is possible considering that these secondary genitalic structures are species-specific and have evolved at rapid rates (Eberhard 1985). The gonopod structures that are potential candidates for stimulation are those that rub back and forth against females *in copula* (in a similar sense to the teeth and spines of several butterflies; Lorkovic 1952, Scott 1978, Platt 1978). Small structures of this kind that are exposed during copulation avoid the high costs that are associated with larger secondary sexual characters can stimulate cryptic female choice. These internal courtship devices may indirectly serve in paternity assurance. A prediction generated from Fisherian runaway is that promiscuous mating by females correlates with genitalic complexity and copulation behaviour (Dixson 1987). The more elaborate spines and prominent sternite of *C. inscriptus* are a fitting case (Chapter 5).

Hence a distinction has been made between the sensory and mechanical functions of gonopods (see also Tadler 1993). This separation generates testable hypotheses centred around cryptic female choice and sperm competition, respectively.

Gonopod function in sperm competition

Centrobolus gonopods are equipped with structures suggestive of dual functions in sperm transfer and sperm displacement. They may not be dual functions *per se* (*sensu*. Waage 1979), but rather separate structures that function in transfer and displacement. The second joint of the anterior gonopods possess lateral lobes that may facilitate the movement of sperm. The distal lamellae of the phallopods are the primary suspects of sperm displacement. They fall into the range of morphological devices that actuate sperm displacement (Eberhard 1985, 1996) and can be categorized as scoops and spines. Scoops have also been recognised on the distal regions of odontopygid and spirostreptid gonopods (Barnett 1997). Spinescent hairs also occur on the distal regions of the gonopod coxites in the millipede *Orthoporus pyrhocephalus* (Barnett *et al.* 1993). Spinescent structures of this sort can displace sperm in the libellulid dragonflies by performing a 'scouring' effect (Miller 1991).

If the gonopod spines in *C. inscriptus* do serve a sensory function, they may benefit males in several ways. Seating the gonopods, detecting previously inseminated rival ejaculates, discovering the point of oviduct emergence and positioning sperm closer to the sperm stores are all behaviours that males can perform to increase favourable sperm distribution and ultimately fertilisation success (*sensu*. Barnett *et al.* 1995).

The structural differences between the gonopods provide secondary evidence for rapid divergence in already complex genitalia. The five classical hypotheses for the evolution of gonopods are not mutually exclusive in this context and possess their own inherent circularities. Thus a carnival of comparative tests have to be performed. In order to do this the female morphology needs to be examined (next chapter) and the copulatory mechanics needs to be reconstructed from functional morphology (chapter 5). The complex morphology and species-specificity of *Centrobolus* millipede gonopods provides part of what

is necessary to examine the copulatory behaviour associated with conflicting sexual interests.

Female sperm storage, capacity and control

Female morphology, reproductive physiology, and behaviour determine the playing field on which males must compete, and many of the rules by which they must play. The female's physiology imposes both physical and temporal constraints on the male's ability to direct sperm towards the ova. The role of the female sperm storage organs in genitalic evolution is well understood in terms of the morphometric constraints they place on sperm positioning (Alexander *et al.* 1997, Hellriegel & Ward 1998, Otronen *et al.* 1997). Under conditions of sperm competition when the female mates with rival males in quick succession, it may be adaptive to redirect the sperm away from her ova when mating is costly. Sperm storage also involves ensuring their survival if they are to be used for fertilization at a later stage.

Centrobolus female millipedes can store the spermatozoa transferred during copulation for long periods (Snider 1981, Hopkin & Read 1992) in a specialized organ, the spermatheca (Wigglesworth 1965). Viable sperm have been found in the spermathecae of female spirostreptidan millipedes that were mated in the previous season (Barnett 1997). *Centrobolus* female morphology is described with the intent to (1) identify the actual site of sperm storage and discern the stage in the evolution of the sperm storage organ. Separation of the spermathecae from the oviduct and the further separation of the sperm insemination duct from the oviduct are recognised as two stages in the evolution of sperm storage organs (Eberhard 1985); (2) count the number of spermathecae. Single and multiple spermathecae both allow female control over different male ejaculate in different ways

(Hellriegel & Ward 1998); (3) remark on how the structure of the spermatheca may affect sperm priority patterns and last male sperm precedence. Predicting sperm precedence from the shape of the spermathecae is a complicated matter (Ridley 1989); (4) examine the arrangement of muscle tissue and consider female abilities to control the movement and use of sperm. Female morphology can be used as evidence that "Cryptic Female Choice" (CFC) is widespread (Eberhard 1996); (5) test if the female is in a race to surrender or accede selectively. Selective acceptance structures include pits and grooves rather than active rejection structures such as erectable spines (Huber 1994, 1995, Arqvist & Rowe 1995). The structure of the *Centrobolus* sperm storage organs are compared to other millipedes and the structural implications for sperm competition are discussed.

Materials and Methods

The females of four pachybolid millipedes ($n = 5$), *Centrobolus inscriptus*, *C. anulatus*, *C. fulgidus* and *C. ruber*, were sacrificed in ethyl acetate killing jars within 24 h of mating with a single male. The anterior regions of their reproductive tracts were removed by cutting away the integument between the second and third diplosegments and pinching the junction between the oviduct and the bursa copulatrix. Dissections were immediately placed onto glass slides and viewed with a Wild M400 light microscope fitted with a camera back. Additional specimens were stored in 70% ethanol (EtOH) before histological sectioning. On removal from EtOH, a single specimen of each species was selected based on the quality of the dissection. The bursa copulatrix was embedded in paraffin wax (melting point 56-60 °C). Sections (1-3 μm thick) were cut on a rocking microtome, stained in haematoxylin/eosin (Humanson 1962) and mounted on glass slides in DPX (Gurr). Photomicrographs were taken under bright field with a Leica Lab K microscope fitted with a camera back. Additional vulvae were removed from EtOH, dehydrated and prepared for

SEM (Chapter 2). SEM micrographs were produced on Cambridge S200 and Leica 440 scanning electron microscopes. SEM was not used extensively, because following an examination of the spermathecae under light microscope and the serial thin sections, no interspecific differences were noticeable.

Results

No interspecific differences in the external structures of the vulva and bursa copulatrix were apparent under the light microscope. There were also no differences between oviducts and associated tissues from stained slides. Thus the female reproductive morphology is only described for *C. inscriptus*.

General vulva morphology

The opaque nature of the female reproductive tract allowed the form of the major components to be distinguished through the vulva membranes (Figure 4.1). The vulvae are simple invaginations of the body wall that open behind the second pair of legs (Plate 4.1A). Within each vulva is a single bursa copulatrix.

Bursa copulatrix

The bursa copulatrix (henceforth bursa) is a laterally compressed structure with a chitinous hinge that articulates two plates. Access to the bursa could only be gained by severing the connecting tissue and pulling the plates apart. The surface of these plates is scale-like in appearance with the occasional seta and pit (Plate 4.1B). The most distinctive features are the short row of approximately ten hairs that point inwards, in a similar manner to those on the leaf apex of the Venus fly-trap (Plate 4.1C). The arrangement of these hairs makes it the most putative tactile site (Plate 4.1D).

The anterior opening of the bursa is covered by an operculum, which when displaced, reveals the opening of the oviduct. A comparison of sections from the upper and lower regions of the bursa revealed that the sperm insemination duct is separate from the oviduct (Plate 4.2A). The insemination duct or furrow tapers further down to a narrow duct, that at one point leads into a single spermatheca ampulla (Plate 4.2B).

Spermathecae and spermatozoa

The duct leading from the insemination furrow of the bursa is narrow (Plate 4.2B). The sperm storage organ occupies less than 10% of the bursa volume. This estimate is based upon the success of serial thin sectioning in bisecting it. The sperm form a compact motionless mass in the distal portion of the long tubular spermatheca (Plate 4.3). The spermatheca is surrounded by bursa gland cells. The general association of the gland cells, rather than one or two layers surrounding the spermatheca, means that their nutritive function is purely speculative.

Muscular tissue

Three different sets of muscle tissue, or "*muscles de la bourse*" (Brölemann & Lichtenstein 1919), were identified. One set fans outwards from the tissue surrounding the oviduct and connect with the bursa plates (Plate 4.2C). Another set fan out in opposite directions connecting the spermatheca to each bursa plate. These are otherwise known as the "*gouttiere apodematique*". A heavily sclerotized region in the lower insemination duct is the confluence site for a third set of muscles. The cross section shows a structure of multiple muscle insertions (Plate 4.2D), hence it was named the "pincushion", and also because its sensory function (if any) remains untested (A. Tadler, *pers. comm.*).

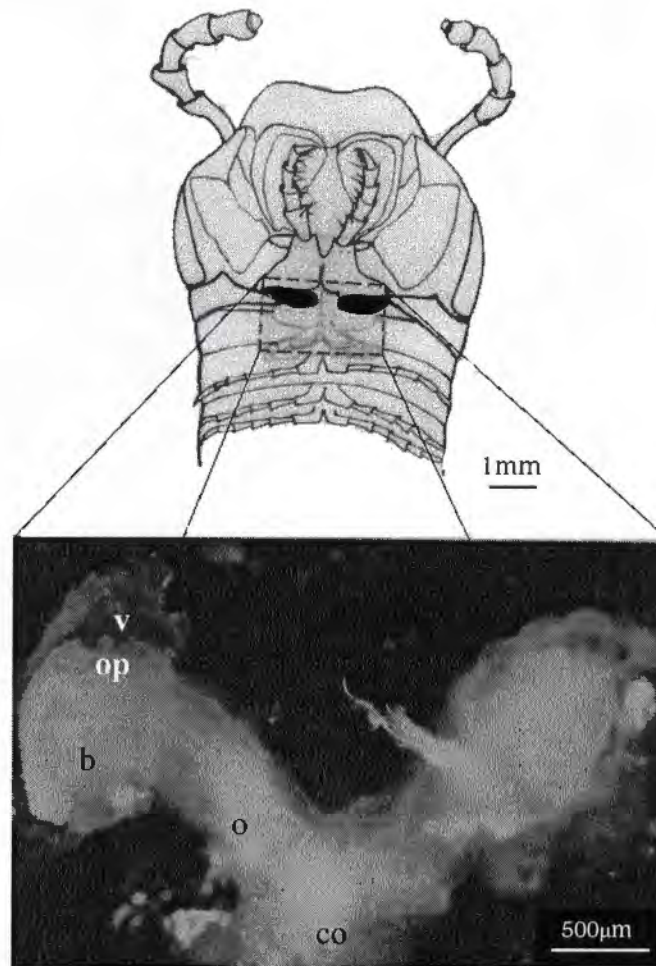


Figure 4.1 Ventral view of the anterior region of the female millipede *Centrobolus inscriptus* (above) with the upper reproductive tract dissected out to show the vulva (v), bursa copulatrix (bc), oviduct (o), common oviduct (co) and operculum (op).

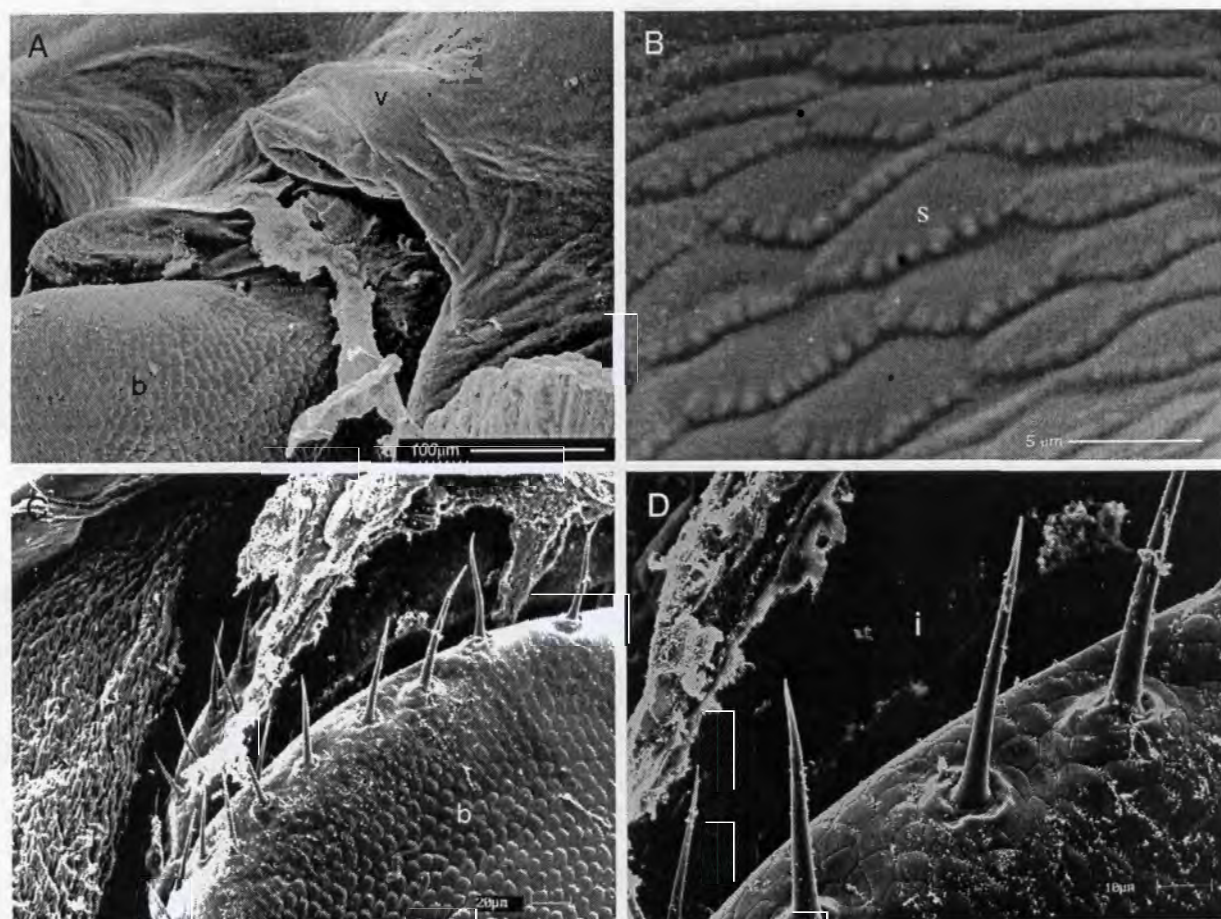


Plate 4.1 Female reproductive tract of the spirobolidan millipede *Centrobolus inscriptus*.

A. Folds of distendable vulva tissue (v) that can be stretched by male appendages during copulation; B. The surface of the bursa comprises overlaid scale cells (s); C. The bursa copulatrix (b) with hairs running alongside the opening between two chitinous plates, D. The inward orientation of the hairs when the furrow or insemination duct (i) is accessible.

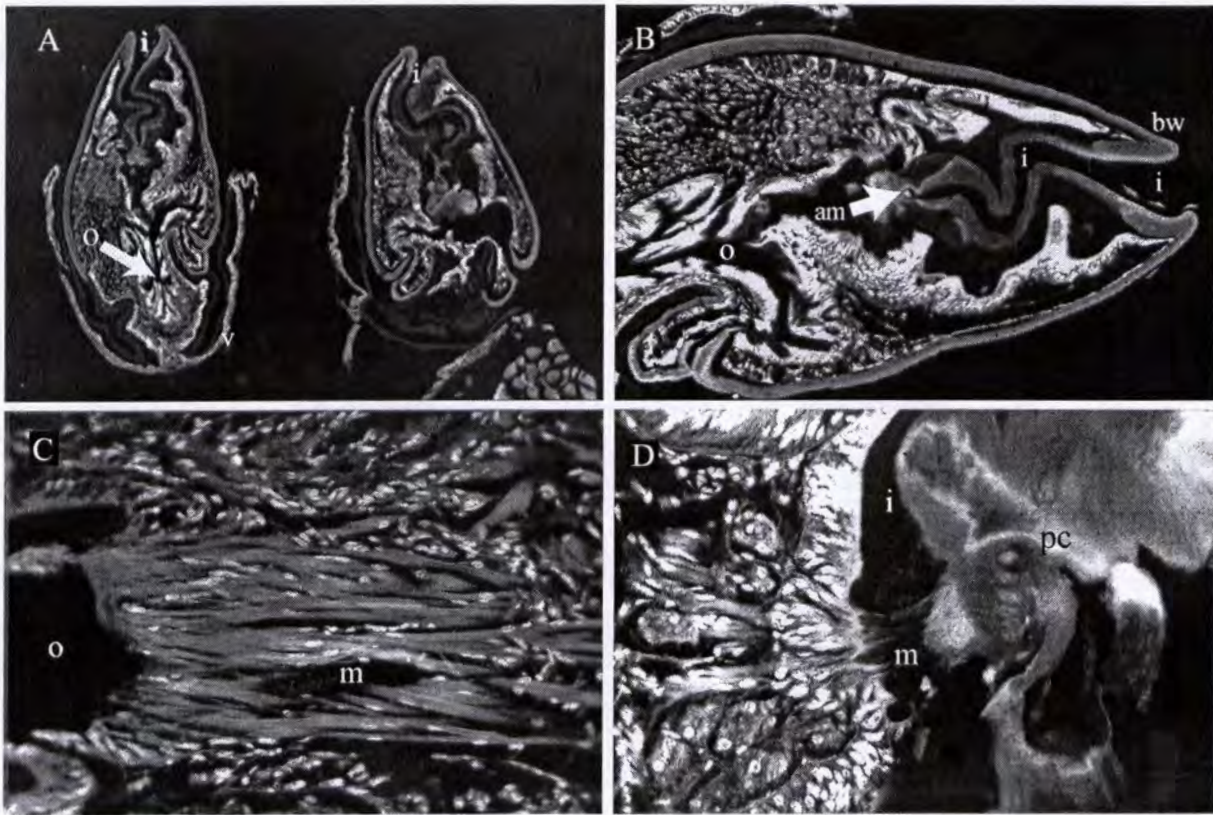


Plate 4.2 Cross sections through the bursa copulatrix of the spirobolidan millipede *Centrobolus inscriptus*. A. Left and right bursae showing the insemination duct or furrow (i) and the oviduct (o) (40X); B. The bursa has two plates with chitinous walls (bw). The insemination duct leads down to the duct of the spermatheca ampulla (am) (100X); C. Muscle tissue (m) connecting the oviduct with the bursa wall (400X); D. Muscle tissue running across the insemination duct and connecting a "pincushion" (pc) (250X).

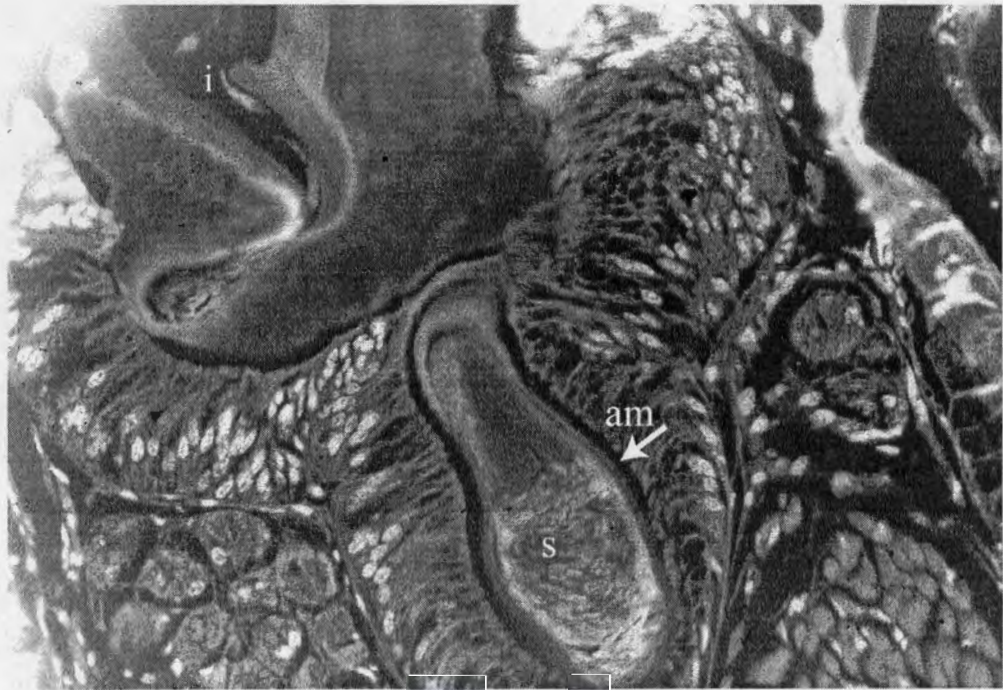


Plate 4.3 Cross section through the bursa copulatrix of the spirobolidan millipede *Centrobolus inscriptus* showing that the insemination duct is separate from the spermathecal ampulla (am) where the sperm (s) are stored.

Discussion

The general form of the female reproductive tract is conserved at the genus level. Thus the histological analysis of the bursa of *C. inscriptus* is taken to represent the general architecture of others in the genus. The distendable properties of the vulva membranes allow the male genitalia to penetrate and perhaps contact the sperm storage organ during copulation. The sperm storage organ of *Centrobolus* can be placed at the second stage of sperm storage organ evolution: the insemination duct, spermatheca, and oviduct are all separate (Eberhard 1996). This stage II is probably conservative in the juliform millipedes, possibly through co-evolution with indirect sperm transfer by the gonopods.

Temporary or permanent: storage and precedence

The insemination duct provides a temporary sperm storage site for when the single spermatheca is full or when ejaculate inseminated exceeds the capacity of the spermatheca. Both ejaculate volume and the time that it spends in this temporary sperm store determine its fate. Larger ejaculate volumes may fill both temporary and spermathecal sperm stores. The length of time that the sperm are stored will depend on: (1) whether it was initially positioned in the spermatheca or the temporary store. Spermathecal sperm stand higher chances for fertilization if oviposition is delayed; (2) sperm competition. When sperm competition is high the chances for displacement from the temporary sperm store increases faster than indirect displacement (e.g. sperm flushing) from the spermatheca; (3) the ability of the female to control sperm movement. If sperm movement between the temporary and permanent sperm stores is regulated then sperm precedence may be either short-term (temporary precedence) or long-term (permanent precedence). The bursa of the damselfly *Calopteryx splendens xanthostoma* (Siva-Jothy & Hooper 1995) and the oviduct of the bruchid beetle *Callosobruchus maculatus* (Eady 1994) illustrate how temporary sperm

storage sites can affect male fertilization success.

Muscling consequences

In *Centrobolus*, the plates of the bursa articulate proximally so that it may function as a prehensor to receive sperm, by mechanical "gate opening" (Eberhard 1985: 105). Once sperm are received they immediately occupy the temporary store. The sperm are saucer-shaped, aflagellate and do not pass through metamorphosis (see also Warren 1934 for a detailed description of the spermatozoa of *C. ruber*). Consequently, all movements of these non-motile sperm must be affected by female processes, whether there is male stimulus or not (Linley & Simmons 1981, Eberhard 1996).

The proposed role of the "pincushion" is for regulating the passage of sperm from the temporary store into the spermatheca. Contraction of pincushion muscles will close the route to prevent entrance as well as exit. Relaxation will free sperm to be moved elsewhere. Provided the spermathecal muscles antagonise the pincushion muscles, sperm will be drawn into the spermatheca when the former contract. Contractions via these fanning muscles, connecting the spermatheca to the wall of the bursa, would cause pumping contractions that in turn would cause the spermatheca to open and allow ejaculate entrance via the furrow (Seifert 1932). Similarly, closing would cause an expulsion of ejaculate outward. The existence of this type of storage system was shown by the cutting of spermathecal muscles in the boll weevil *Anthonomus grandis* (Villavaso 1975). A severed spermathecal muscle does not impair spermathecal filling yet prevents sperm exit and reduces displacement from 66% to 22%. Predicting sperm precedence from the shapes and sizes of sperm storage organs (Walker 1980), is no longer possible because of complex reproductive tract morphology (Hellriegel & Ward 1998).

Are insemination, fertilization and oviposition all decoupled?

The eggs develop in paired ovaries within a common median oviduct that runs from about the 15th diplosegment to the last podous ring (Hopkin & Read 1992). The mature eggs travel in single file along the common median oviduct which bifurcates anteriorly into two short oviducts. This initial sequence of events is standard in most orders of millipedes. In *Centrobolus* the oviduct opens alongside the insemination duct so the egg presses on the operculum that opens to release it into the region where the bursa is connected. The eggs have to pass through the temporary sperm store to get released from the vulvae.

The 'egg simulation' hypothesis (Tadler 1996a) needs testing if we are to verify that insemination and oviposition are coupled. The prediction is that the male gonopods stimulate the passage of an egg through the oviduct to cause (1) mechanical tensions on the basis of the vulva and the connection between the bursa and the operculum, and (2) removal of sperm from around the opening of the spermatheca, i.e. the insemination duct. The presence of a fully developed egg in the bursa immediately after mating (*pers. obs*) provides preliminary evidence in support of egg simulation in *Centrobolus*.

The route that the egg takes and when it eventually gets fertilized depends on the relative volumes of ejaculate in the temporary sperm store and the spermatheca. When the temporary store is full then fertilization is coupled to ovulation. However, when the insemination duct is empty and the only sperm are within the spermatheca, then insemination, fertilization and ovulation, can all be decoupled. If this is the case then the female may have absolute control over fertilization. Muscle fibres that connect the oviduct with the plates of the bursa indicate that peristaltic contractions are possible. Peristaltic contractions of the oviduct presumably serve to regulate egg release depending upon optimum fertility periods (Austin 1975).

Somehow the release of sperm from the spermatheca would have to be co-ordinated to meet the arrival of the egg. It seems as though the egg is protected from fertilization by the operculum covering the opening of the oviduct into the bursa copulatrix. In the fly *Musca domestica*, the egg gets protected from fertilization by a cap on the micropyle (Leopold *et al.* 1978). The cap is removed when the micropyle is positioned at the mouth of the spermathecal duct, ensuring that sperm from the spermatheca have precedence over that from the bursa.

The general association of the bursa glands surrounding the spermathecae suggest that they may have a nourishing function. Prolonged storage of sperm has been associated with specialized cells and nutrient provisioning (Michener 1974, Kaulenas 1992, Barnett 1997). Insect sperm usually retain their fertilization capacity throughout much of, if not their entire reproductive life with the aid of nutrients (Davey & Webster 1967). Presuming that females benefit from prolonged oviposition then the permanent sperm precedence is predictable.

Accessibility of previous sperm to the current male

The stage II sperm storage with a single spermatheca places constraints on last male sperm priorities. Males have full access to the temporary sperm stores, provided that the bursa is kept open and that the sperm remain there for long enough periods after the last mating. The opportunity for direct sperm removal from the spermatheca is denied since the inlet duct is narrower than the distal lamella of the male gonopod. In many diplopods, the lumina of the receptacula or spermatheca is too narrow for male gonopods to access (Brölemann & Lichtenstein 1919, Verhoeff 1926-1932 cited in Hopkin & Read 1992, Kurnik 1988, Tadler 1993, Barnett 1997).

Sperm displaced from the temporary sperm stores can still affect displacement from the spermatheca by a flushing process. Therefore a current male that inseminates larger volumes and employs flushing using self-sperm may trade-off temporary sperm precedence with permanent sperm precedence. This is similar to employing a 'Loligo strategy' of flooding the oviduct with sperm (Eberhard 1996), and perhaps even effecting fertilization before the female is ready to oviposit.

Sensory structures

The spines on *Centrobolus* gonopods were said to function in seating the gonopods, detecting previously inseminated rival ejaculates, discovering the point of oviduct emergence and positioning sperm further into the sperm stores (Chapter 3). Considering the gonopods as having a sensory function in detecting the point of oviduct emergence is the most appropriate in the context of egg simulation. A full corroboration of this hypothesis is also suggestive of the coadaptation of male and female sensory structures.

The hairs on *Centrobolus* bursae resemble basiconic receptors. Sensilla that innervate the "pincushion" may produce a stimulus that results in spermatheca and oviduct muscle action. Male gonopods are perhaps capable of stimulating the female in this region, inducing a message to be sent to the pincushion. If this scenario is true, then egg simulation can be expected. The "pincushion" could be equivalent to the "*poil sensoria*" (Brölemann & Lichtenstein 1919), however the function of both is still unclear because no transmission electron microscopy or electrophysiological investigation has ever been performed (A. Tadler *pers. comm.*). The hairs of the bursa resemble basiconic receptors that also occur in the odonate *Enallagma glaucum* (Robertson & Paterson 1981). In *E. glaucum*, the sensilla spread out over a groove that occupies the male genitalia. Male gonopod movements are

predicted to cause the female to draw sperm into the spermatheca as well as simulating the release of eggs.

In conclusion, (1) the actual site of sperm storage is inaccessible to direct sperm removal but larger ejaculate volumes deposited into the temporary sperm store may flush rival sperm to achieve permanent sperm precedence; (2) A single sperm store of *Centrobolus* indicates that the potential for the female to influence relative fertilization success of males is only possible by insemination site mortality (Hellriegel & Ward 1998). If the second male can access the actual insemination duct of the bursa through the furrow (next chapter), then the entire ejaculate contents can be removed from the temporary sperm stores; (3) The spermatheca is distendable and its shape has no bearing on male sperm precedence; (4) After the mechanical “gate-opening” of the bursa and insemination, all directional sperm movement is under the influence of female muscle action. Muscles with sensory innovations (pincushion) may antagonise the spermathecal muscles causing sperm to be drawn into the spermatheca when the pincushion contracts, but expelled when it relaxes. (5) It is not clear whether the structures of the bursa place the female in a race to surrender or accede selectivity. This physiological perspective on the intricacies of female sperm storage organs and their controlling mechanisms may serve as a turning point in predicting active sex roles.

Sensational copulatory mechanics: male ‘functional luxuries’ make the right genital connections

Waage (1979) made a breakthrough by showing a “Dual function of the damselfly penis: sperm removal and transfer.” The dual function of male genitalia in sperm displacement and transfer has subsequently been predicted from the combined examination of the ultrastructures of the male and female genitalia (Siva-Jothy 1985). It is a widespread fact that not all male genitalic structures function only in sperm transfer during the time of copulation (*in copula*), but that they perform copulatory courtship through movements and interactions with the female genitalia (Eberhard 1985, 1996). These structures can be considered ‘functional luxuries’ because they can induce cryptic female choice by stimulating structures on the female genitalia, while facilitating rival-sperm displacement and sperm transfer.

Most knowledge of genital mechanics is based upon field observations of copulating pairs and genital morphometrics. Genitalic complexity is probably underestimated in many species because they have only been studied in the retracted or relaxed state (Eberhard 1985: 8). Historically, where animal genitalia have been examined in an everted state it has been exclusively for taxonomic purposes (polygyrid snails, Webb 1947; snakes, Dowling & Savage 1960; Klauber 1972; Myers 1974; spiders, Comstock 1967; lacewing insects, Tauber 1969; plecopteran insects, Picker 1980; cydnid bugs, Dolling 1981; rays, Ishiyama 1967). A ‘functionless luxury’, as opposed to a ‘functional luxury’ is defined as a genitalic structure that has no further function after male and female genitalia have coupled (Huber 1994). Fortunately, in some species it is possible to freeze-fix mating pairs *in copula* so as to

establish how the genitalia of both sexes function. Sexual behaviour and genital mechanics can be well understood by freeze-fixation of genitalia *in copula*, e.g. in arachnids (Huber 1993, 1994, 1995) and various diplopods (Tadler 1996a, Barnett 1997). The congruent fixation of the coupled genitalia is the only way to test hypotheses centered around the coadaptation of male-female genitalia yet it is rarely used.

The genital biomechanics of *Centrobolus* millipedes are examined *ex copula* then *in copula* to make actual correlations between the male and female genitalia. The aims here are to determine (1) whether the contact zones between male and female genital structures are within reach of the sperm stores. Sperm competition requires the introduction of at least part of the male genitalia into the female sperm storage organ (Waage 1979). In most animals the males genitalia do not reach deep enough into the female to directly manipulate stored sperm (R. Smith 1984, Eberhard 1985, Birkhead & Moller 1992, Eberhard 1996). A prediction is that the structures of the male genitalia that contact the female are those that differ most between species (Chapter 3); (2) what genitalic movements are performed syncopulation. Genitalic movements can induce female responses and consequently increase male reproductive success (Dewsbury 1988); (3) the specific structure-function relationships of the male gonopods, female vulvae and the coupled structures. In spirostreptidan millipedes, a single genitalic component (telopodite) could function in ejaculate placement, repositioning and displacement, and female stimulation (Barnett 1997). This is truly a 'functional luxury'; (4) whether the male gonopods interact with the sensory regions of the bursa copulatrix. Tactile or sensory structures in the contact zone can function in cryptic female choice (Eberhard 1996).

Materials and methods

Artificial simulation of gonopod movements

Gonopods of the four species, *C. inscriptus*, *C. anulatus*, *C. ruber* and *C. fulgidus*, were removed from freshly killed males and manipulated under the light microscope. The coleopods were pinched distally and pulled outwards so that the movement of the phallopods could be produced by slowly opening the gap between the anterior and posterior gonopods. The insertion of a pin behind the coleopods causing the phallopods to extend allowed them to become dehydrated in that position. One specimen from each species were chosen and prepared for SEM. The sequence of gonopod movements were reconstructed on the SEM micrographs using Adobe photoshop 3.0 and Illustrator 7.0.

Freeze-fixation

Five copulatory pairs of *C. inscriptus* were formed by introducing single males to females in plastic containers (13cm diameter). After copulating for approximately 10 minutes pairs were freeze-fixed by pouring liquid nitrogen (-196°C) upon them. After one month in 70% ethanol at -10°C the copulatory organs were dissected from the animals under the light microscope to remove the vulva sacs and associated tissue that concealed the region of contact. The cleanest specimen was chosen and prepared for SEM.

Results

The male genital mechanics in three species of *Centrobolus* were successfully simulated by artificially manoeuvring the anterior and posterior gonopods (Plates 5.1 - 5.3). In these species, the gonopod complexes could be positioned in a relaxed or retracted state and then brought to an everted state. These basic movements involve the basal region of the coleopod moving in an anti-medial fashion so that the phallopod extends aborally. The distal lamellae

moves in an arc so that it becomes the structure extended the furthest from the body.

Observations of *C. inscriptus* prior to fixation revealed that the female vulva was pulled out from the vulva sac by the grasping knob and bill of the coleopods. Once the gonopods were seated they became inflated and erectile. After the pair were fixed, three significant contact zones between the male and female genitalia of *C. inscriptus* were recognized (Plate 5.4). (1) The distal telopodite of the coleopod, armed with spines, presses on the inside of the vulva; (2) The phallopod inserts the bursa copulatrix and pulls on the anterior junction between its two plates so that the insemination duct or furrow is exposed; (3) The sternite fits between the legs of the female so that the genitalia of the two sexes are symmetrical (Plate 5.6).

The structure-function relationships of the male gonopods (Table 5.1) indicate that there are two processes involved in genitalic coupling. Stage I involves the coleopods and stage II the phallopods. The coleopods have a mechanical function that provides the phallopods with deeper access. Stage II involves the phallopods positioning within the bursa. Both pairs of gonopods were seated symmetrically within the female vulvae.



Plate 5.1 Scanning electron micrograph illustrating the male genital mechanics of the millipede *Centrobolus anulatus*. The phallopod (p) articulates from the base of the coleopod and extends in an aboral direction. The distal joint of the coleopod (d) opens sideways in an anti-medial fashion, while the basal joint (b) remains static. In this specimen the phallopod has been fully extended to reveal the distal lamella (dl), the structure that interacts directly with the female genitalia during mating. The movement of this gonopod complex (arrows) is from a retracted towards an everted state. The forward motion is proposed to function in sperm competition through its displacement activity

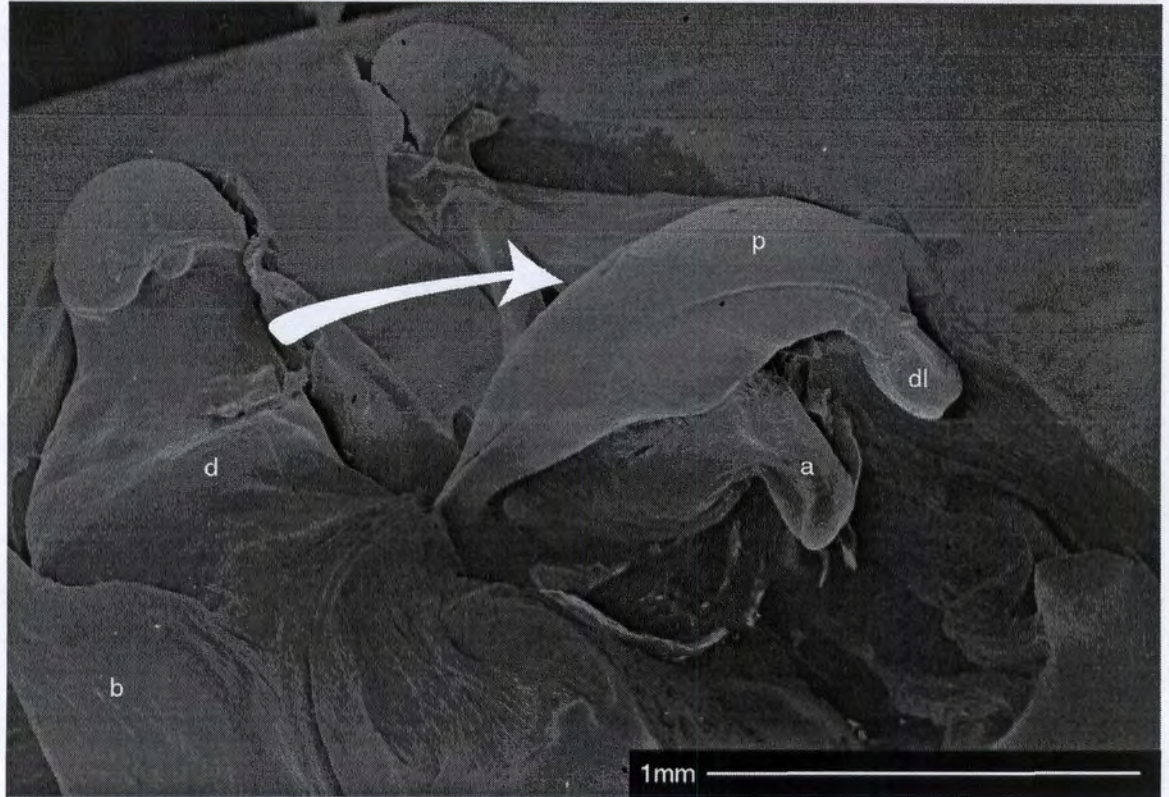


Plate 5.2 Scanning electron micrograph illustrating the male genital mechanics of the millipede *Centrobolus ruber*. The right phallopod (p) is fully everted with its distal lamella (dl) extended to extreme and the basal lobe (a) in an inflated state. In this species the coleopod, with its basal (b) and distal (d) telopodite joints, remains static rather than shifting laterally.

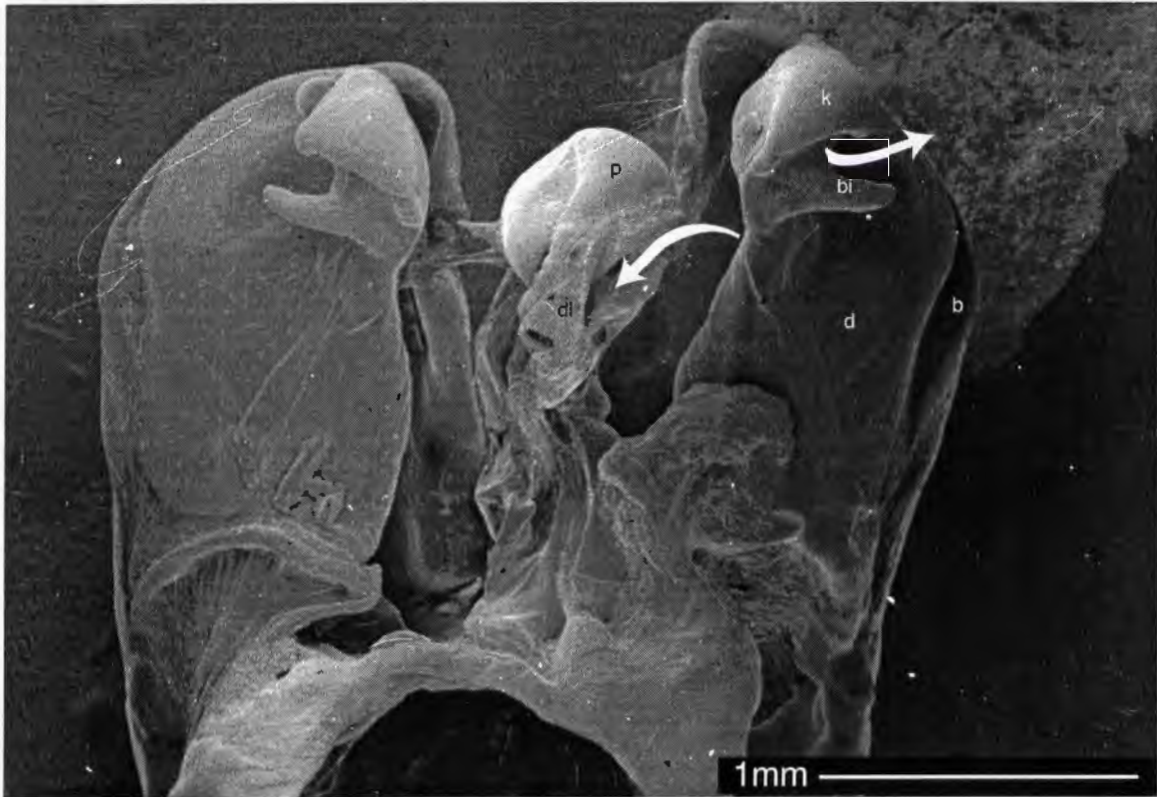


Plate 5.3 Scanning electron micrograph illustrating the male genital mechanics of the millipede *Centrobolus fulgidus*. The right gonopod (on the left) is in a retracted state while the left gonopod is everted. The phallopod (p) slides out from between the distal (d) and basal (b) telopodite joints of the coleopod so that the distal lamella (dl) remains furthest from the body. Simultaneously, the knob (k) and the bill (bi) of the coleopod shift laterally in an anti-medial fashion. The movement of this gonopod complex (arrows) is towards the everted state, revealing the gap between the anterior and posterior structures that is proposed to enhance sperm displacement.

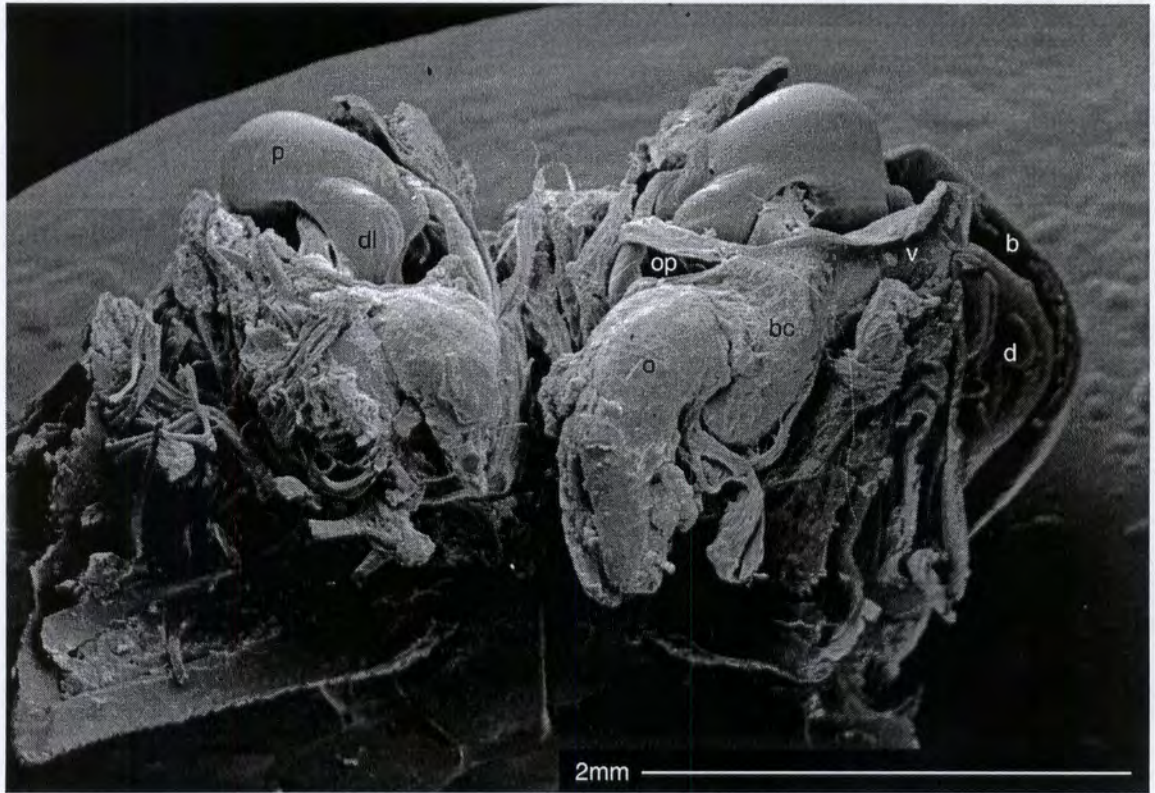


Plate 5.4 Scanning electron micrograph of *Centrobolus inscriptus* male and female genitalia that were freeze-fixed *in copula*. Notice that the distal lamella (dl) of the posterior male intromittent organ, the phallopod (p), is seated near the operculum (op) of the female sperm storage organ, the bursa copulatrix (bc), which leads into the oviduct (o). The distal joint of the telopodite (d) of the anterior intromittent organ, the coleopod, is shifted anti-medially to distend the female vulval sac (v) while the basal joint of the telopodite (b) remains stationary.

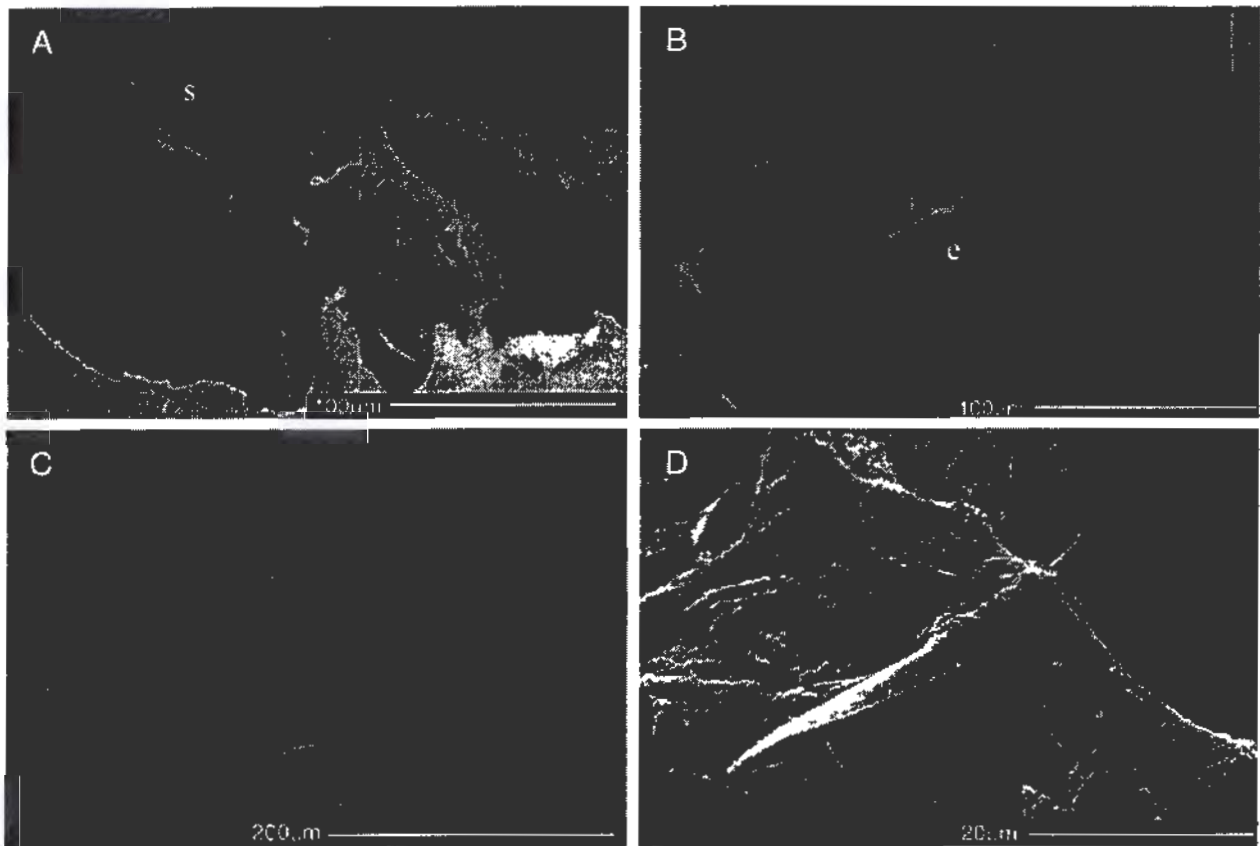


Plate 5.5 *Centrobolus inscriptus* regions of genital contact that illustrate where the male gonopods redistribute ejaculate and where the female sensory structures are bent. A. The bill (bi) is a distal process with slightly curved grasping spines (s) facing in different directions; B. The spines are capable of independent movements, rubbing against the female and redistributing ejaculate (e); C. The distal lamella (dl) of the male phallopod inserts the female bursa copulatrix (bc) in a distal region covered with long hairs similar to basiconic receptors; D. A single hair on the bursa showing its resemblance to the insect basiconic receptor and its directional movement with passing ejaculate.

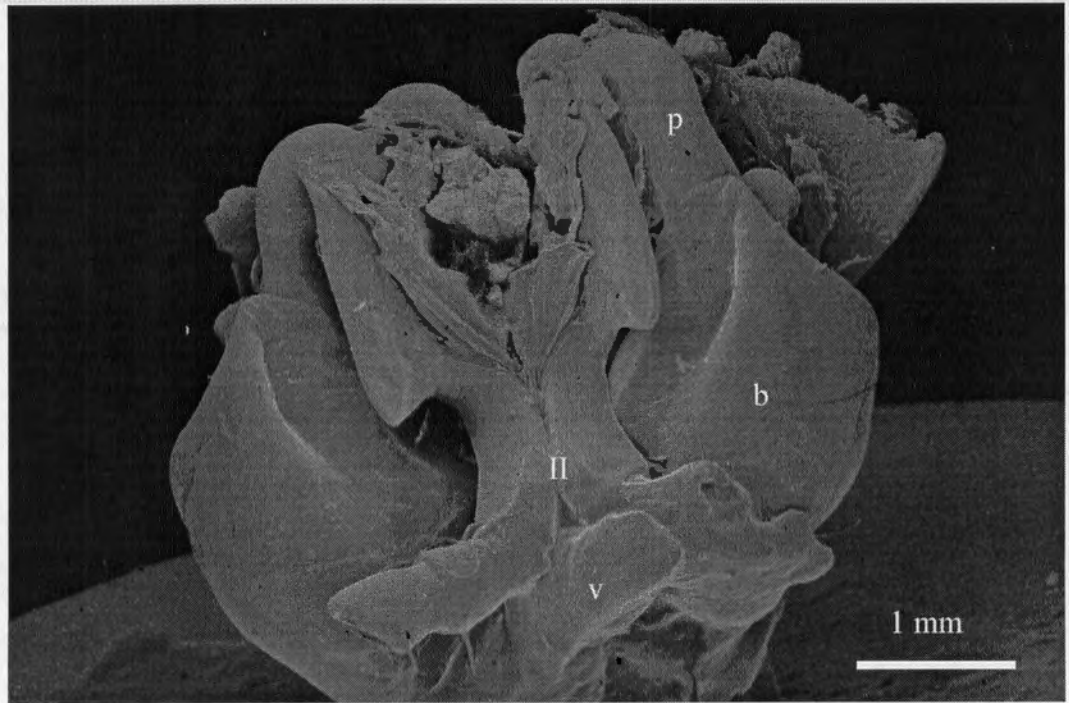


Plate 5.6 An oral view of the freeze-fixed genitalia from a pair of *Centrobolus inscriptus* in copula. The male gonopods remain firmly locked within the vluvae, the sternite (v) rests between the females second pair of legs (II), the basal joints (b) are inflated outwards, the phallods (p) are also inflated to give the gonopod complex a two-fold reach.

Table 5.1 Speculational structure-function relationships of the male accessory genitalia (gonopods) in the spirobolidan millipede genus *Centrobolus*.

Gonopod structure	Function	Functional equivalent
Coleopods	protective when retracted	julid promerite
	stretches the walls of the vulvae apart	
(1) distal telopodite	prevents slipping (i.e. mechanical)	clasping projection of the
	facilitates seating (i.e. sensory)	cheirite in <i>Craspedosoma</i>
	places the gonopods (positioner)	<i>transsilvanicam</i> (Tadler 1993)
(2) sternite	prevents lateral shifting (stabiliser)	
	stretches the vulva sac in a medial plane	
Phallopods		julid solenomerite
(1) sperm groove	directs sperm transfer (inseminator)	
(2) distal lamella	enters the female bursa (see text)	

Discussion

Various contact zones between male and female genitalia provide evidence that the male genitalia are more complex than simple casts. The female genitalia do not simply contain the male gonopods. The coupling of male-female genitalia in *C. inscriptus* urges a discussion of mutual mechanical co-adaptation between genitalia, but first the functional roles of both male and female genitalia must be clarified.

Dual functioning genitalia

It is known that there has been a transition in the evolution of spirobolidan gonopods from proterospermous (sperm transfer by anterior gonopods) to opisthospermous (sperm transfer by posterior gonopods (Berns 1968). In *Centrobolus* the coleopods can be classed as paragonopods or peltogonopods because they play a subsidiary role as inseminating devices. As accessory plate-like structures their function is in protecting the gonopods proper. The phallopods are the gonopods proper that actually transfer the sperm.

The distal lamellae of the phallopods do not enter the spermathecae as is also the case in spirostreptid and odontopygid millipedes, so direct ejaculate removal from the permanent sperm stores is mechanically impossible (Barnett 1997). The end-plates and distal processes of the phallopods may function to remove rival male sperm from the temporary sperm stores and reposition self sperm closer to the spermatheca and oviduct. Thus *Centrobolus* gonopods possess two of the five structures with putative functions in sperm displacement that are known in other millipedes (Tadler 1996a): (1) various projections on a moveable component (telopodite); (2) flagellae (and pseudoflagellae); (3) brushes; (4) end plates; as well as (5) tips on a distal process (opisthomerite).

The insertion of the phallopod into the bursa confirms that the males have access to the temporary sperm stores, that there is an exit, and that the gonopods have the opportunity to perform movements *in copula*. The orientation of the sperm groove into the bursa illustrates that sperm transfer and displacement may occur dualistically. Hence the phallopod is a functional luxury.

During stage I of *Centrobolus* gonopod, while the coleopods are stretching the vulvae, the phallopods evert. The process is best described as a hydropneumatic system, as discovered in the chordeumatid millipedes (Tadler 1993). The phallopods become fully erectile when inflated by muscular action during stage II. Muscles that induce pressure changes are manifest by movements of specific structures or segments (Siva-Jothy 1985). Thus there is movement of the phallopods within the female genital tract after coupling has been accomplished.

Comparative functional morphology of millipede genitalia

The current results closely parallel those of Tadler's (1996a) study of the genital apparatus in four julidan species of millipede, *Nemasoma varicorne* (Nemasomatidae), *Brachyiulus lusitanus*, *Unciger foetidus* and *Cylindroiulus boleta* (Julidae). *C. ruber* most closely resembles *N. varicorne* and *B. lasitanus* in that the vulvae remain within the vulva sac during copulation. The gonopods of *C. inscriptus* are functionally more similar to *U. foetidus* and *C. boleti* than the two aforementioned julidan species where parts of the gonopods form clasper-like structures that pull out the female vulvae from the vulva sacs.

Centrobolus gonopods have functional equivalencies with the "lower" julids. The former possess coleopods and phallopods while the latter possess promerites and opisthomerites. The absence of an additional intermediate structure separates both of them from the "higher

julids" that possess the mesomerite (Verhoeff 1928, Attems 1941). The most obvious functional equivalent is between the julid solenomerite and the *Centrobolus* phallopod, both the sperm transferring structures which are introduced into the central funnel leading into the receptaculum seminis or bursa, respectively. This is similar to the functional morphology of *B. lusitanus* and *C. boleti* where "a projection of the posterior gonopods (end-projection, brachite) fits into a slit anterior to the openings of the receptacula." (Tadler 1996a). A minor difference is that only the distal lamella of the *Centrobolus* phallopod enters the female bursa. This may be realised through genitalic recognition or stimulation if a male-female contact zone of significantly smaller surface area were to be responsible for sperm destinies.

Centrobolus and its allies may constitute a relict group which possesses some lower julid characters. Thus their gonopods could represent the plesiomorphic condition of juliforms, however the speciose nature may be due to minor variation in complex genital structures. The major obstacle to using any comparative approach to trace the evolution of genitalia is the difficulty in identifying the homologous structures (see attempts in arachnids, Huber 1995; in odonates, Miller 1981). The problem is that extremely high functional divergence of homologous structures operate in the mechanism of genitalic coupling (Huber 1994).

Unending evolutionary chase

In *Centrobolus* the tips of the elaborate and species-specific genitalia reach into the areas of the upper regions of the female reproductive tract where sperm are stored temporarily but are prevented from passing through the insemination duct into the oviduct and sperm storage organ. This means that direct male-male competition is only possible within the insemination duct (Common 1970). The deep-seated bursae with long insemination ducts

and furrows in the female vulvae (Chapter 4), and the correspondingly long phallopods of the male gonopods, suggest an unending evolutionary chase (*sensu* Wilson 1988). Most studies use measurements of male genitalia length and female genital tract length to ascertain how closely they correspond (e.g. in Odonata, Siva-Jothy 1985). Congruent measurements of this kind are only estimates because they do not account for the elasticity and folds of the cuticle and associated female tissues, in combination with the inflation and erection of male genitalia.

Unlike the unending evolutionary chase that is set in a conflict of interest, a confluence in male and female interests may happen by the time that the phallopods are inserted. During this time, female millipedes are able to perceive male genitalia and to discriminate between minimal morphological variants in two ways. Female discrimination may be based on a mechanical fit or sensory structures (Tadler 1996*b*).

Mechanically fitting mate choice

In *Centrobolus*, the male genitalia seem to have exceeded the limits imposed by body size with a naturally selected innovation in the form of a folding and hydraulic gonopod mechanism. This 'hydropneumatic' mechanism has increased the mechanical complexity of the genitalia (Tadler 1990). In most animals, the evolutionary change of male genitalia is constrained by the limits of male body dimensions. In millipedes, allometric relationships with the gonopods are complex and many species have large and elaborate gonopods (Telford & Dangerfield, *unpubl.*).

Sensory mate choice

Hairs on the female bursa make directional movements when contacted by the distal most structure of the male gonopods. These are an appropriate sensory basis for perceiving male

genital stimulation and making an active mate choice. The bursa plates may be held together denying the male access or the right stimulus may produce gate-opening. In *Enallagma glaucum*, the sensilla spread out over a groove, “ideally suited to the recognition of the appropriately convex inner surface of the male [superior] appendage” (Robertson & Paterson 1982). If these are sensory hairs they need not be involved in recognition *per se*, but if they connected to the female “pincushion”, equivalent to the “*poil sensoria*” (Brölemann & Lichtenstein 1919), then cryptic female choice can be controlled by the complex female muscle actions (described in the last chapter).

The functional morphology provides the necessary prerequisite for evaluating behavioural mechanisms by sperm competition, cryptic female choice, and conflicts in mating interests. The accessibility of the male genitalia to the temporary sperm stores are expected to enhance sperm displacement. The architecture of female genitalia, with a single sperm storage organ, a temporary sperm store, and antagonistic muscles, allow for control of directional sperm movement between stores. This division between temporary and permanent sperm storage may cause a conflict of interests between the sexes. Males striving to achieve permanent sperm precedence may capacitate a sperm store or misdirect sperm “travel” into sites when it is useless in fertilization that produces optimum oviposition. All structural interaction between male and female genitalia suggests coadaptation, however this may change depending on the copulatory behaviour and differential control of sperm movements between individuals of a copulating pair.

Engagements in sexual conflict, endearing male and enduring female mating strategies

The form and resolution of sexual conflict in the evolution of insect and arachnid mating systems is currently under hot debate (Choe & Crespi 1997). A listing of the costs and benefits, both phenotypic and genotypic, for males and females to prolong copulation shows a growing realization that their primary interests in matings are asymmetric (Dickinson 1997). Orthodoxy has it that the male always benefits from remating while the female experiences the associated costs. The majority of benefits accrued by the male are the same as those achieved through maximizing copulation numbers, i.e. increased paternity assurance (Stockley 1997), new ideas are being provided for why a female remains together with a male and maintains genital contact for a prolonged period. Hence the female mate-guarding hypothesis (Eens & Pinxten 1995, Finke *et al.* 1997).

In order to fully establish the adaptive behaviour of the female, it is imperative to recall initially the male perspective (Rodriguez 1994*b*). Prolonged copulation is a behavioural adaptation that benefits males by reducing sperm competition (Parker 1970). The costs, in terms of reduced mating frequency due to reduction in the time to locate and fertilize multiple females, reduced survival due to loss of time spent feeding, increased conspicuousness to predators, or reduced ability to escape from predators, are low in terms of the chance to increase paternity. Thus in male-female encounters, a conflict over the control of the mating decision typically occurs as these contact guardings may be costly to females.

In *Centrobolus* millipedes, both males and females have evolved morphological characteristics that appear to aid in forcing and resisting copulation, respectively. Males possess tarsal pads for grasping females (Chapter 2) and various genital processes that function to hold the female anteriorly (Chapter 5). Females have spines on the bursa copulatrix that may function to exclude males from their sperm stores (Chapter 4), and are known to engage in 'recoiling' and a reluctance to mate (Tadler 1993). The aim here is to (1) identify the sequence of behaviours that take place before, during, and after copulation; (2) quantify variance in copulation duration. In millipedes copulation is usually prolonged and differs between species and between populations (Berkowitz & Warburg 1988, Telford & Dangerfield 1990a, Barnett 1997). Attention is given to intraspecific variance in copulation duration and the conflict between males and females in terminating copulation. Although males can control copulation duration in some species (Telford & Dangerfield 1994), there may be a conflict of interests. The conflict of interests hypothesis predicts that there is also a female "giving-up" time before copulation becomes costly; (3) test for size related copulation duration in males and females. A prediction is that species with prolonged copulation have positive size-assortative mating (Ridley 1989). An accompanying prediction is that copulation duration is shorter when sexual size dimorphism (SSD) increases; and, (4) identify any male produced stimuli as potential mechanisms of cryptic female choice (Eberhard 1996).

Materials and methods

Centrobolus millipedes were collected from their typical habitat in Afromontane coastal forest. A subset of four species were chosen for comparative purposes because they could be easily collected in large numbers and identified by their distinct genital morphology. *C. ruber* is the southernmost of these four species, and therefore also the least tropical; Port Shepstone (30° 42' S, 30° 27' E). *C. inscriptus* and *C. anulatus* are latitudinal intermediates with sympatric distributions; Mtunzini (28° 51' S, 31° 46' E). *C. fulgidus* is the northernmost, a subtropical species; St Lucia (28° 23' S, 32° 25' E). The majority of observations were made during the summer rainy seasons of 1995-1996 and 1996-1997.

Millipede copulatory events form a continuous sequence and because copula pairs rather than precopulatory behaviours are mostly encountered in the field, it was decided to collect large sample sizes and return to the laboratory to reconstruct the full behavioral sequences from continuous observations. Copulation movements were video recorded in a close-up position using a standard VHS camera to provide detailed descriptions of any copulatory courtship. Behaviour patterns were identified by repeatedly freeze-framing the footage. Positions *in copula* were categorized as being either in parallel or coiled (*sensu* Telford & Dangerfield 1993). Simple descriptive statistics were used to seek covariation around copulation duration. Movements produced by the males *in copula* were also described.

Results

Behavioural sequence

Rapprochement or pair formation involves (a) members of both sexes becoming highly surface active and “swarming” as a result of external stimuli brought about by single showers of rain, (b) a quicker response in males moving at approximately 1.5 times faster

(1.6cm/sec, $n = 10$) than the females suggesting them to be the searching sex. This was more so the case for *C. inscriptus* with surface activity shifting from the forest floor vertically upwards into the trees and shrubs (c) females possibly producing long range pheromonal and close range cutaneous attractants. After finding a mate the mounting stage involves the male running up the back of the female and clinging to her aided by the tarsal pads on the legs (Chapter 2). Relatively long precopulatory periods, up to 10 minutes, would ensue if the female conglobated (recoiling in the same manner as that in response to attack from predators) before the male could obtain access (Plate 6.1A). A male would often attempt to reduce the female resistance by attempting to uncoil her using the labrum (mouthparts), first three pairs of legs, and semi-everted gonopods. The female's reluctance to mate sometimes involved the withdrawal and eversion of the vulvae.

Physical coupling is a multistage process. It seems to be a matter of time before the male uncoils the female and when the male has succeeded in unrolling the female, the two sexes align and make ventro-ventro contact. The ventral surfaces of both are contiguous with ring seven of the male opposite ring three of the female (Plate 6.1B). The head of the female is held in a fixed position by the first three pairs of the males legs. The gonopods evert and the female vulvae, opening on segments two/three are pulled out by the male coleopods on ring seven confirming the speculated function of the coleopods (Chapter 5).

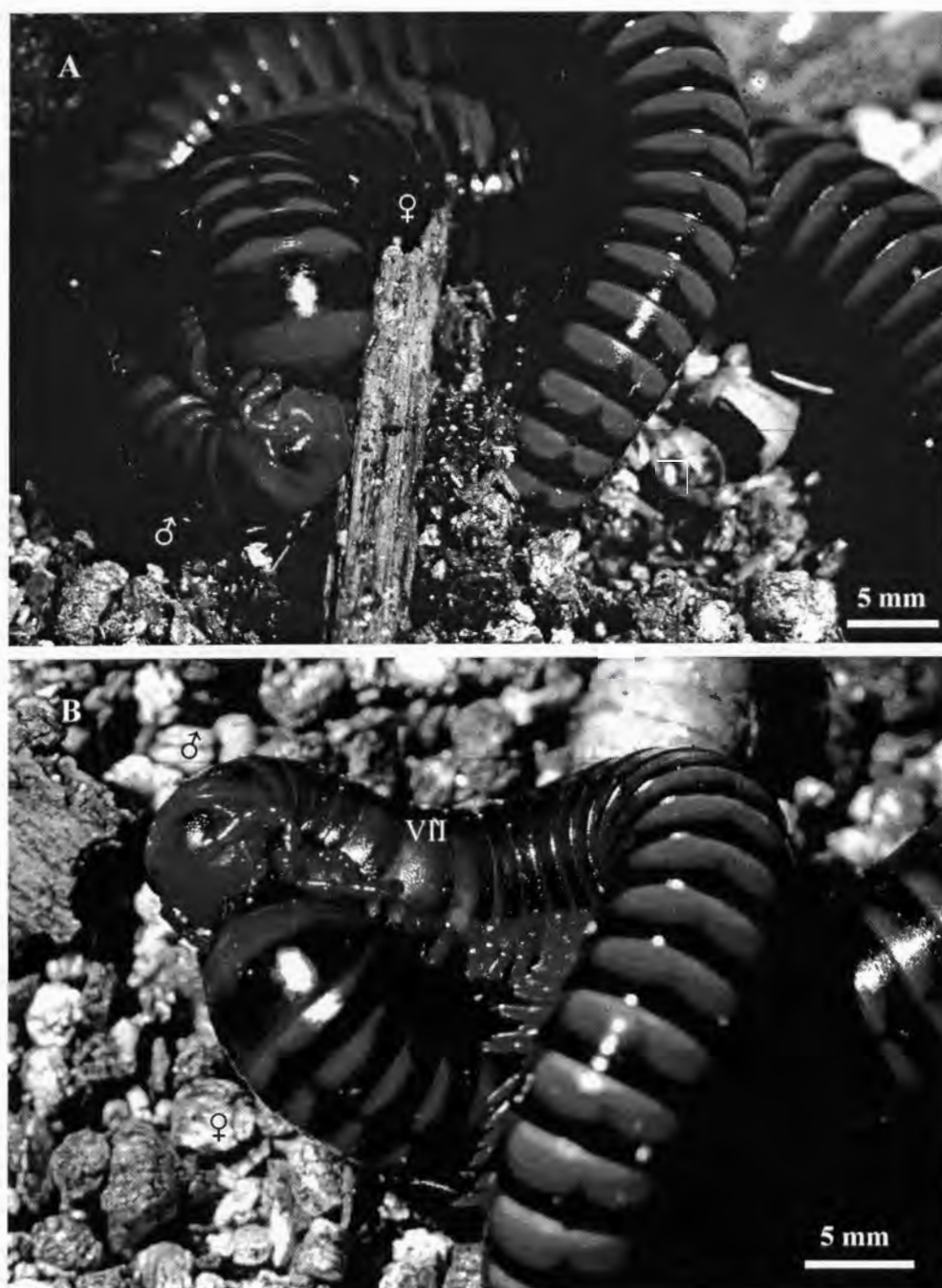


Plate 6.1 The initial stages in the mating sequence of the millipede *Centrobolus inscriptus*. A. The precopulatory struggle begins with the female denying the male access by conglobating (coiling up) but soon results in the male uncoiling the female; B. The male, with the trunk twisted around the female, is in the process of everting the gonopods on the seventh (VII) diplosegment.

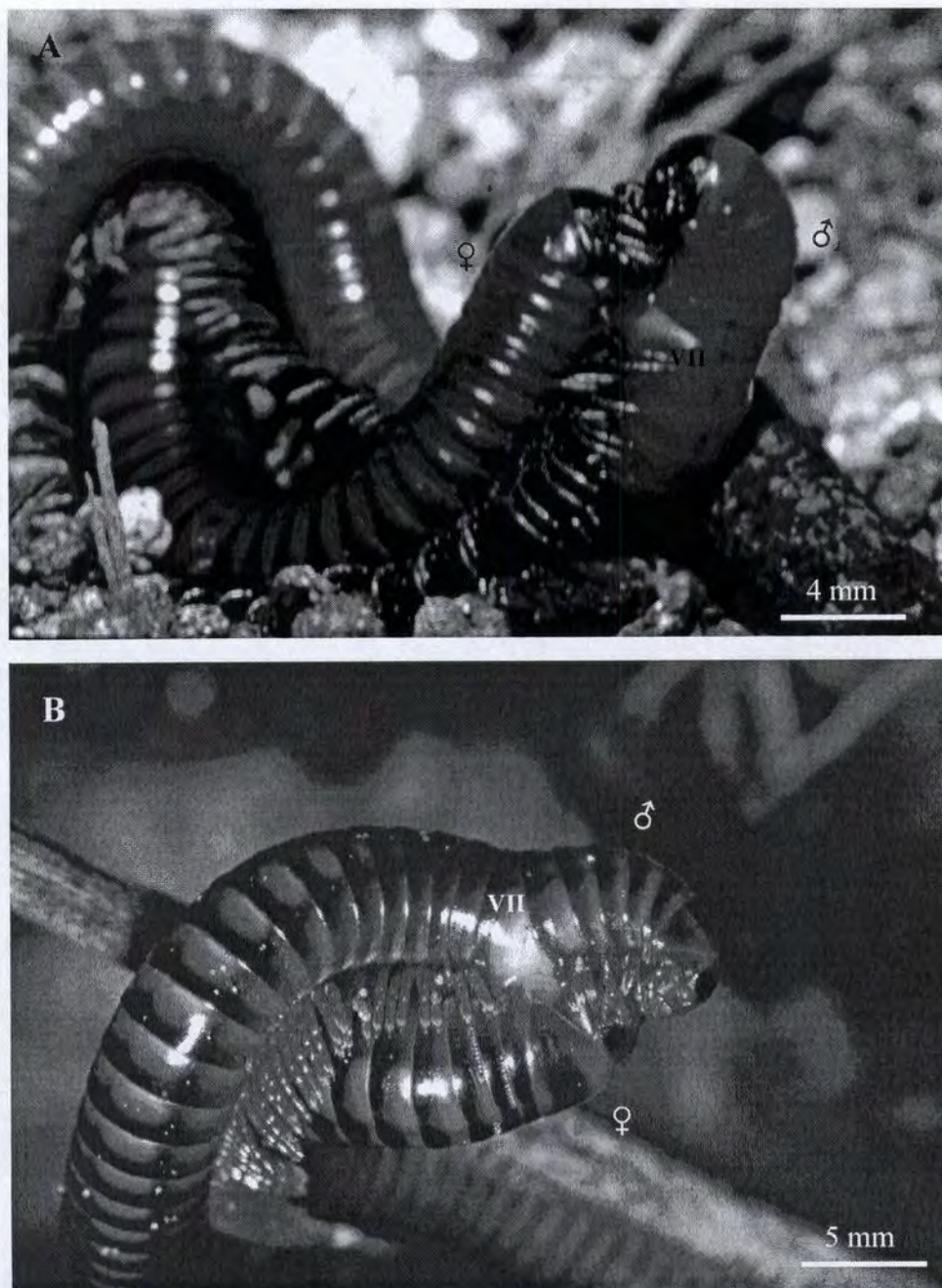


Plate 6.2 The copulatory positions of two millipede species differing in the extent to which the gonopods are everted and enter the female vulvae: A. *Centrobolus ruber* showing that the gonopods are shallow-seated; B. *C. inscriptus* gonopods are completely everted and deep-seated with the resultant production of the sexual 'collar' that is typified by hydropneumatic movements associated with bouts of sperm transfer and displacement. The membranes of the gonopod-bearing seventh (VII) diplosegment evert and inflate to form a sexual collar.

Positions in copula and the sexual collar

Genital engagement occurs as the male inserts both pairs of gonopods into the female and conducts rhythmic movements, presumably to seat the gonopods symmetrically. The gonopods may be inserted and removed from the female up to ten times. It is hypothesized that this initial period is associated with both insemination and displacement (see Chapter 7). Once firmly established, genital contact was maintained throughout copulation. The region of genital contact displayed hydropneumatic movements as the membranes of the gonopod-bearing seventh diplosegment everted and inflated to form what will henceforth be termed the “sexual collar”. The sexual collar is pronounced in *C. inscriptus*, present yet less hydropneumatic in *C. fulgidus* and *C. anulatus*, and almost invisible in *C. ruber* (Plate 6.2). The end point of physical coupling was different between species. Positions assumed *in copula* were either in parallel or with the male coiled around the female (Plate 6.3).

Postcopulatory behaviour

The disengagement of the male from female genitalia often results in a vigorous pulling apart with the male gonopods still everted. Disengagement of the gonopods takes the form of a “snap-release” in *C. inscriptus*, which is not nearly as vigorous as that seen in the Spirostreptidae, but more so than *C. ruber* where the pair passively disengage. This is followed by genital grooming in both sexes. The male retracts the gonopods and moves away first. The female takes longer before she too leaves the site of copulation.

Durations of copulations (syncopula)

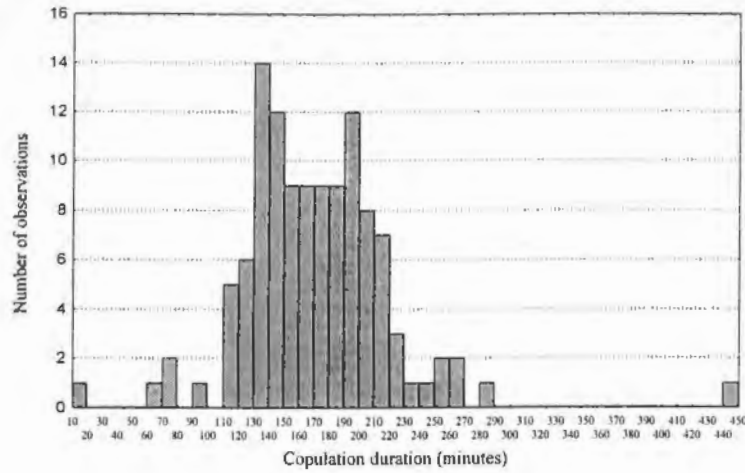
In four species of *Centrobolus* millipedes there was considerable variation and overlap in the duration of copulation (Table 6.1).

Table 6.1 Variations in copulation duration of four spirobolidan millipedes of the genus *Centrobolus*.

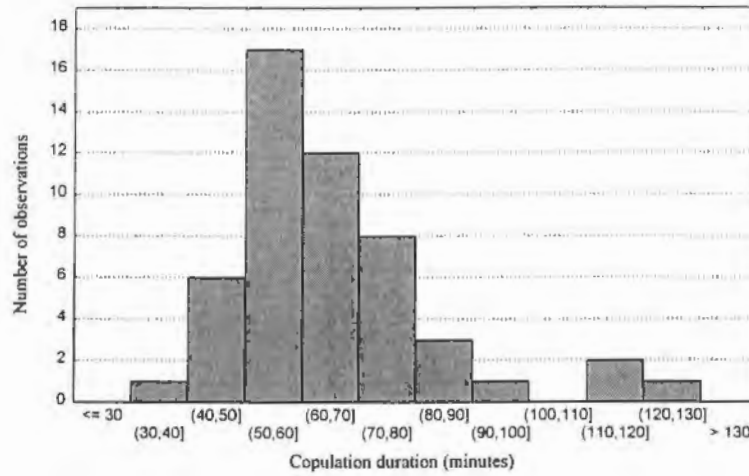
Species	Copulation duration/min	<i>n</i>	Coefficient of Variation
	(Mean ± SD)		(%)
<i>C. inscriptus</i>	170 ± 49.3	115	29.0
<i>C. fulgidus</i>	66.4 ± 18.6	51	28.0
<i>C. ruber</i>	39.8 ± 13.2	32	33.1
<i>C. anulatus</i>	39.4 ± 18.6	8	47.3

If the distribution of copulation duration for *C. inscriptus* is bimodal (Figure 6.1 A) it may represent an intersexual conflict of interests. Each peak may be the optimum copulation duration for the different sexes, with the longer second peak that of the most controlling sex. The skewed distribution for *C. fulgidus* (Figure 6.1 B) indicates the possibility of strong directional selection for longer copulations that are controlled by members of one sex. The normal distribution of *C. ruber* (Figure 6.1 C) looks as if copulation duration is under stabilizing selection, but one cannot say whether one sex or the other is in control. The two atypical copulations that were far beyond the extremes, one 440 minutes long was a pair that attempted yet failed to disengage suggesting male control, and the other only 15 minutes long was possibly an adult male that attempted to mate with what was a lower stadia female.

(A)



(B)



(C)

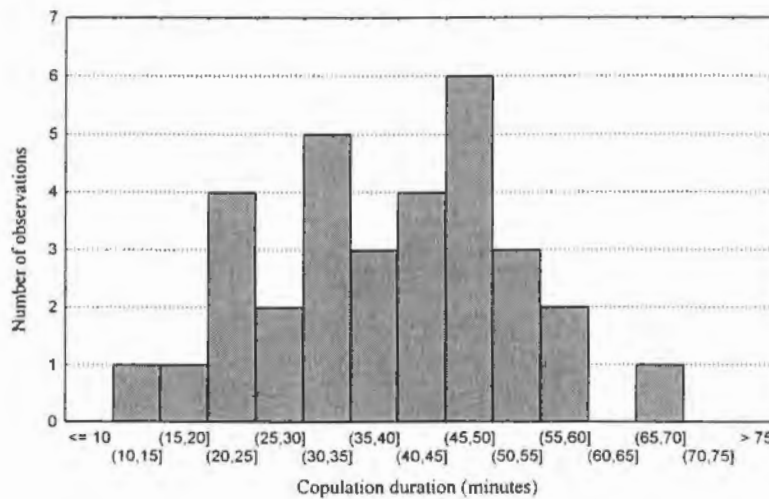


Figure 6.1 Frequency histograms of the copulation durations recorded from populations of three spirobolidan millipedes; (A) *Centrobolus inscriptus*, (B) *C. fulgidus* (C) *C. ruber*

Copulation duration and body size

Larger females tended to copulate for longer than their smaller conspecifics in *C. inscriptus* ($r = 0.63$, $n = 30$, $p < 0.05$) which was not the case with males (but see the following chapter).

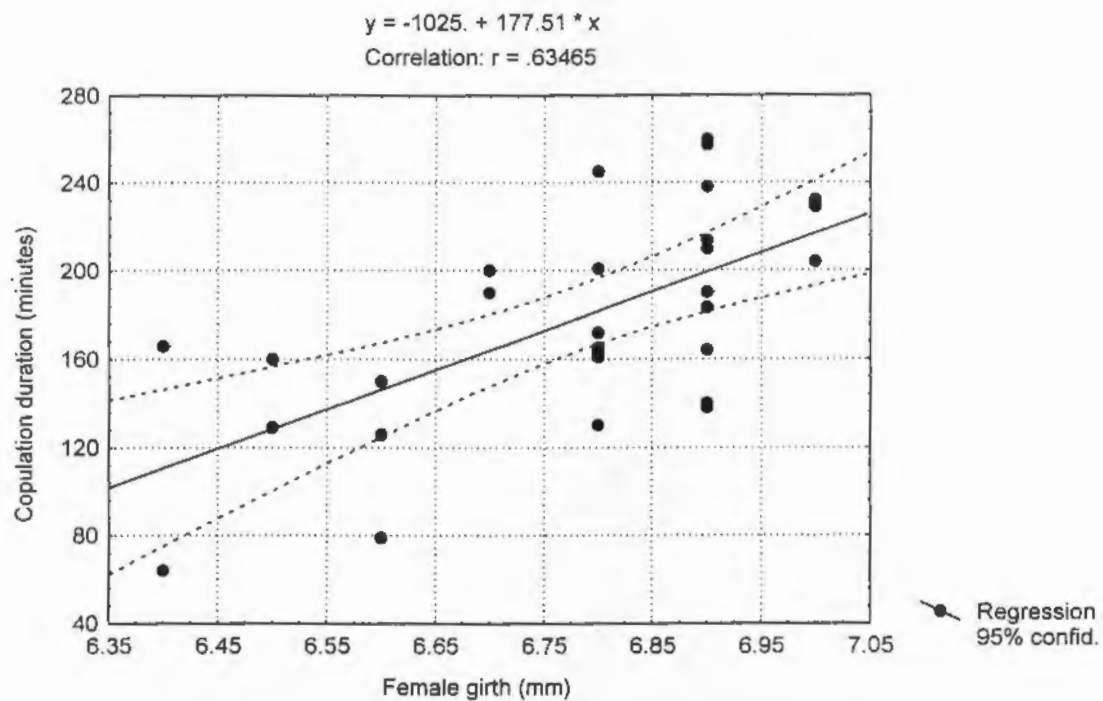


Figure 6.2 Regression of copulation duration on female body width in the millipede *Centrobolus inscriptus*.

Table 6.2 Copulation durations recorded in non-spirobolidan species of Diplopoda

Species (geographic locality)	Copulation duration (minutes)	<i>n</i>	Position in copula	Reference
Spirostreptidae				
<i>Alloporus uncinatus</i> (Hwange)	205.8 ± 60.8	25	coiled	Telford & Dangerfield 1993
(Mazowe)	122.7 ± 49.4	35		
	97.9 ± 39.4	65		
<i>Calostreptus</i> sp. (Hwange)	60.3 ± 25.6	25	parallel	Telford & Dangerfield 1993
(Mazowe)	33.8 ± 22.9	25		
<i>Orthoporus pyrrhocephalus</i>			coiled	Barnett 1997
Odontopygidae				
				Telford & Dangerfield 1993
<i>Odontopyge</i> sp. 2 (Marondera)	66.0 ± 11.2	28	coiled	
<i>Odontopyge</i> sp. 3 (Vic. Falls)	80.2 ± 25.3	20	coiled	Telford & Dangerfield 1993
<i>Odontopyge</i> sp. 3 (Marondera)	85.6 ± 16.6	19	coiled	Telford & Dangerfield 1993
<i>Spinotarsus</i> sp. 1 (Marondera)	92.3 ± 18.6	19	coiled	Telford & Dangerfield 1993
<i>Chaleponcus digitarsus</i>	38.7 ± 25.3	20	coiled	Barnett 1997
Harpagophoridae				
<i>Poratophilus dipodontus</i>	23.7 ± 10.3	250	parallel	Barnett 1997
Parajulidae				
<i>Aniulus bollniani</i> (N. America)	20 - 30	5	parallel	Mathews & Bultman 1993
Craspedosomatidae				
<i>Craspedosoma transsilvanicum</i>	116 - 463		coiled	Tadler 1993
Julidae				
<i>Ommatiulus moreletii</i>	33.5(17-53) min	23	parallel	Carey & Bull 1986
Polydesmidae				
<i>Streptogonopus phipsoni</i>	12 ± 5.2 (max.120)	120	parallel	Schubart 1934, Sahli 1969, Stephenson 1961
	14.5	2		
<i>Polydesma inconstans</i>	120 (max. 24h)		coiled	Mukhopadhyaya & Bahakat 1983

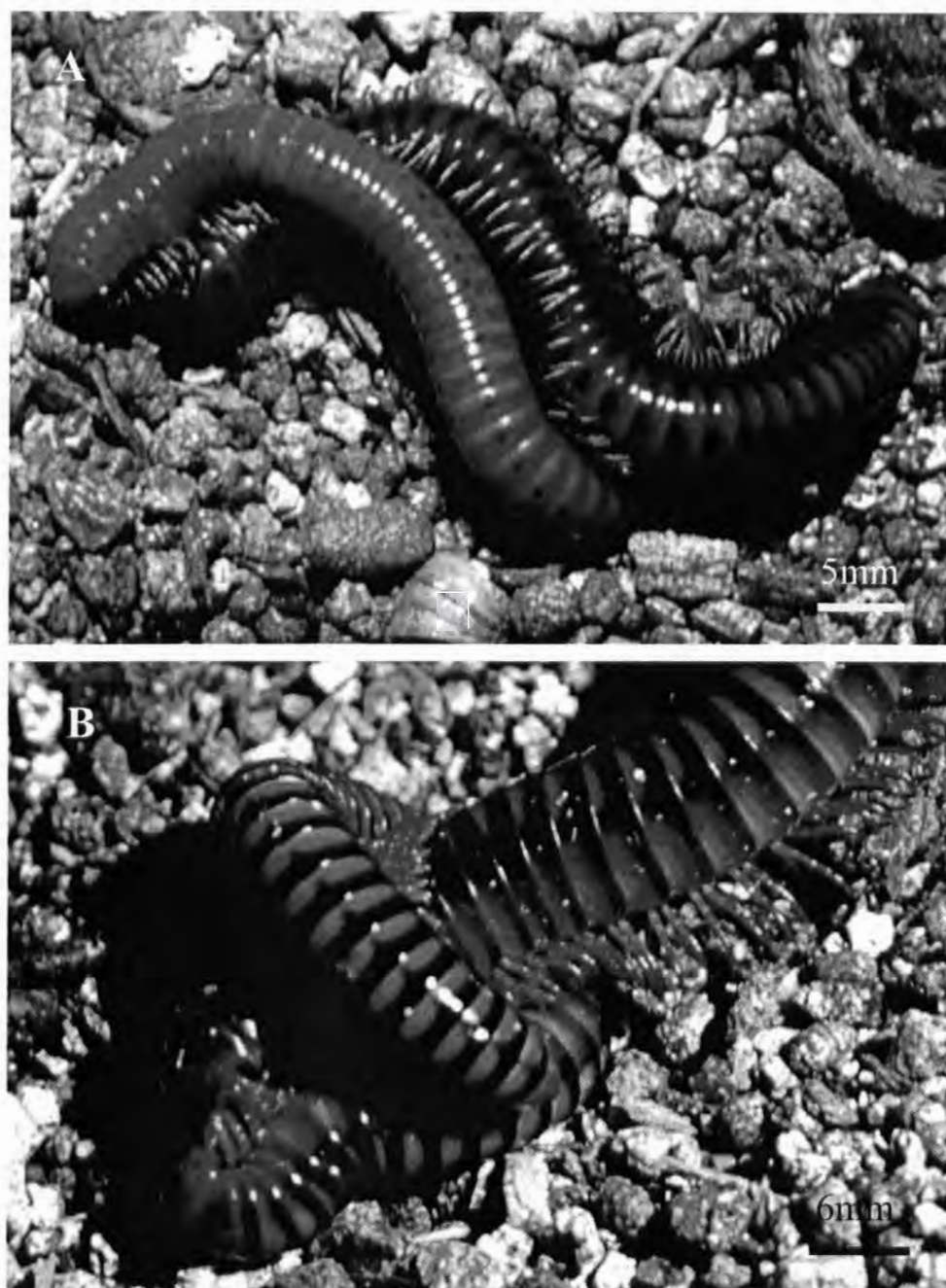


Plate 6.3 Two extreme positions assumed by copulating millipedes:

A. *Centrobolus ruber* is a parallel copulator for a short duration;

B. *C. inscriptus* is a coiled copulator lasting for prolonged periods.

Copulatory courtship

Three courtship behaviours, which could be advertisements of male qualities that bias female choice, were witnessed (Figure 6.3):

(a) The antennae of the male frequently tap on the head of the female with sideward movements around her ocelli, (b) A glandular secretion is produced by the male in the later stages of copulation, emanating from the mandibular region and running across to the female, and (c) the genital collar pumps in a hydropneumatic rhythm.

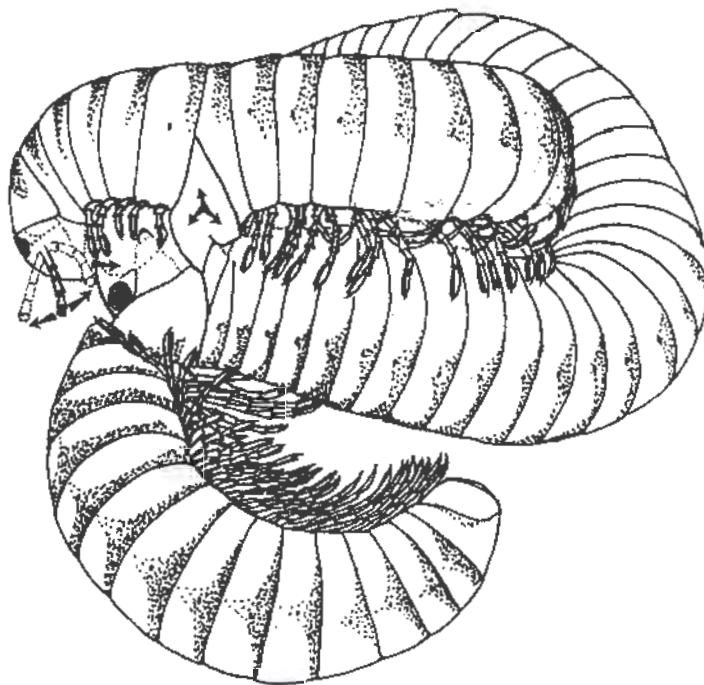


Figure 6.3 Three copulatory courtship behaviours in *Centrobolus inscriptus* that probably serve to advertise qualities that may bias the choice of the female toward the male. (a) Antennating: the antennae tap frequently on the head of the female; (b) Glandular secretion: a watery substance produced from the mandibular region during the later stages of copulation; (c) 'sexual collar': hydropneumatic inflations of the membranes of the ventral surface of the gonopod-bearing seventh diplosegment. Arrows indicate the movement of the antennae, glandular secretion and sexual collar, with dotted lines showing the active positions of a structure.

Discussion

In helminthomorph millipedes the males initiate copulation by approaching the females from behind and moving towards the head along the dorsal surface (Mauriès 1969). The topic of mate recognition and the associated behavioural cues were not investigated and are assumed to follow a random walk (Telford & Dangerfield 1993).

Precopulatory struggle

It is accepted that during this initial period in the mating sequence that the evolutionary interests of males and females differ (Trivers 1972, Parker 1979). Females avoid males by coiling in a manner similar to predator avoidance behaviour (Haaker & Fuchs 1970, Mukhopadhyaya & Saha 1981). Precopulatory coiling by the female is considered a test of male quality or fitness because fitter males are better able to uncoil females (Tadler 1996b).

Alternatively, recoiling may be a case where intersexual selection represents ‘non-adaptive’ female choice because mate selection is a side-effect rather than an ultimate aim of female reluctance (c.f. waterstriders *Gerris odontogaster* Arnqvist 1992). Or, it may be adaptive, given that copulation is somewhat costly and dangerous for a female (Daly 1978). The persistence of the male to uncoil the female can further be explained as a form of ‘sensory trap’ (West-Eberhard 1983, Christy 1995), by attempting to uncoil the female the male is behaving in a similar fashion to a predator. Either way, it remains to be seen whether the presence of the male causes a change in the motivational state that accelerates a “general physiological change to [female] mating readiness, independent of the particular male” (Alexander *et al.* 1997).

The allometry of copulation duration

Two reasons can be given for why larger females endure longer copulations. Larger females endure to control the duration of copulation and benefit from improved fecundity (see Chapter. 2) and fertility. This may come about through maximizing her intake of ejaculate or some other nutrient-rich substance produced by the male; predicting a correlation between ejaculate volume or material benefits with copulation duration (see next chapter). Alternatively, males endeavor to control the duration of copulations and so monopolize the larger and more fecund females, in turn maximizing their paternity assurance. Although millipede copulations can be entirely male controlled (Telford & Dangerfield 1990*b*), it seems doubtful that an individual can assess the body size of a potential mate because of the nature of head-to-head courtship.

Sexual control of copulation duration

The shortest copulation durations were recorded for species which show less vigorous copulation; as is the case in other juliform millipedes (Telford & Dangerfield 1990*a,b*) where differences in the duration of copulation are thought to reflect the intensity of sperm competition between species - prolonged copulation is a form of mate guarding whereby the male attempts to assure paternity by controlling the duration of copulation (Thornhill & Alcock 1983). In odonate species long copulations (minutes-hours) are associated with sperm removal and short copulations (seconds) are associated with sperm repositioning (Siva-Jothy & Tsubaki 1994).

Although no general relationship between the mechanism of sperm displacement and copulation duration has been shown across phylogenetically unrelated taxa, similarities in

genital structures may indicate closer relatedness. *Centrobolus* resembles *Craspedosoma* in copulatory behaviour more so than either of the spirostreptidans *Alloporus* and *Orthoporus*.

Copulatory courtship and cryptic female choice

The active movement of the antennae against the females head during copulation suggests a function in courtship. Sexual selection for antennae is testable if the males antennae are larger than the females and there is fluctuating asymmetry (e.g. Cerambycidae, Møller & Zamora-Muñoz 1997). This area is the most sensual region because of cephalisation in the long cylindrical *bauplan*. The glandular secretion transferred from the male to the female during copulation may be considered as an indirect effort to invest in paternity. Prezygotic nutrient contributions of this kind are a form of mating effort since they function to stimulate the females and secure matings (Alexander & Borgia 1979). Until the chemical content of this substance, and its nutritive role can be established, no conclusion can be made. It can be said that if the actions of the glandular secretions that are produced by the male affect the likelihood of his paternity then courtship has not evolved to its end.

Attempting and preventing take-overs

Sexually active solitary males that encounter a pair already *in copula* attempt to take-over the female but soon give up and form 'triplets'. In the locust leaf-minor *Odontota dorsalis* (Coleoptera: Chrysomelidae), with prolonged copulations and male-biased sex ratios, males also attempt take-overs unsuccessfully and soon form 'trios' consisting of a second male atop a pair (Kirkendall 1984). Males seem to endear in a strategy of associating with the first female that they find because when the operational sex ratio (OSR) is male-biased, encounter frequency with solitary females declines (Telford & Dangerfield 1996). This behaviour is not seen as a consequence of inferior competitive ability linked to an alternative mating strategy

(Krebs & Davies 1987) because *C. inscriptus* even form 'clusters' consisting of two pairs *in copula* and a third male (*pers. obs.*). Copulation 'clustering' suggests that all males engage in this strategy when population densities are high and the OSR is male-biased. Thus it is adaptive to sometimes behave as if mating pairs are inseparable, making few attempts at dislodgement other males they but rather waiting for the *copula* pair to dissociate. In the beetle *Tenebrio molitor*, male genitalic spines that are similar to those on some millipede gonopods function to prevent premature dislodgement during copulation (Gage 1992). The inflated sexual collar and firmly seated gonopods make it very difficult to separate copula pairs and pulling them apart by hand requires much more force than an individual millipede is likely to produce.

The sexual collars-and-ties of endearing male and enduring female strategies

The different patterns of copulation duration displayed in the three *Centrobolus* species indicate how more prolonged copulation and higher levels of sperm competition could have evolved. Initially a population is under stabilizing selection, then the pressure for prolonging copulation as a paternity assurance in males produces directional selection, before a conflict of interests between the sexes may result. The mean copulation duration for a species at any one instant in time should also represent the outcome of a sexual conflict over insemination, depending on the relative ability of males and females to exert their interests over one another (Simmons 1991).

The emerging trends are for more 'endearing' male and 'enduring' female mating strategies to result from a conflict of sexual interests. Endearing males strategies include the ability to win precopulatory struggles, copulate coiled rather than parallel, maximize copulation duration, and produce a prominent sexual collar. Enduring female strategies are those in

direct conflict. Only the reluctance to mate is evident. It is clear that we do not know enough about female behaviours during prolonged copulation. The behavioural patterns that have been outlined can now be combined with the genital morphology to provide a backdrop for the following chapters that will test the outcome of females' prolonging copulation.

Prolonged copulations, male sperm displacement and female sperm dumping in *Centrobolus inscriptus*

There are a myriad male adaptations that have evolved to 'get' sperm to eggs and a myriad more that counter their paths to fertilization. When there is high variance in sperm competitiveness and high selective pressures on males to fertilize eggs, the result is an acceleration in the evolution of these counter adaptations. The physical mechanisms of these counter adaptations include sperm removal and replacement (Parker 1970, Waage 1979), inactivation (Silberglied *et al.* 1984), dilution (Parker 1970, Walker 1980), and many more, which are all male mediated behaviours. Males that endear in these behaviours have higher potential fertilization success, a priority based largely on positioning self sperm over rival male sperm (Schlager 1960, Walker 1980), and will ultimately be the individuals with the higher reproductive rates. Females pay high costs if they are slow to coevolve with these processes associated with male-male competition that can occupy long periods of time.

Prolonged copulations are one adaptation to counter sperm competition that can result in a sexual conflict (Stockley 1997). More complex adaptations have been discovered through investigations into the causal factors of Darwinian sexual selection, invariably those studies that reduce mating processes to gametes. For example in *Drosophila melanogaster*, seminal fluid reduces the competitive ability of sperm from other males but also reduces the females propensity to mate (Rice 1996). When the basic mechanisms of sperm competition remain unchanged at the gametic level from one mating to the next, the female suffers no asymmetric costs, just those which are suffered equally by the males, e.g. predation (Arnqvist 1997). In

monarch butterflies *Danaus plexipus*, ejaculates are broken down but the mass and nitrogen content decrease at constant rates (Oberhauser 1992). When the time required for breakdown of large spermatophores is longer than the remating interval it is competing males that lose, by investing in the offspring of other males, while the female suffers no apparent costs. In cases like these, it is the individuals "time in" and "time out" of the mating arena that is the major determinant of its potential reproductive success. Often the most simple sexual conflict is over which sex is in control of the actual duration of copulation (Rowe *et al.* 1994, Thornhill & Sauer 1991). By withholding the outcome of this one reproductive event, an individual is able to control the future reproductive success of not only itself but also that of its partner.

Females that choose to resolve intersexual conflicts over copulation duration will suffer less from lost time and obtain the chance to accrue benefits by pursuing other activities. The role of sequential female mate choice in species with polygynandrous mating systems that foster sperm competition with males providing no material benefits to the female can be used to answer many questions. Three especially important questions that have been addressed recently are why females mate multiply, what rules do they follow in making a successive choice, and what different criteria are used in the choice (Gabar & Halliday 1997). Females can engage in either a pre-copulatory mate choice or a relative postcopulatory mate choice. The two hypotheses are tested depending on the presence or absence of an interaction between the effects of prior and future mates on the remating interval (Brown 1997). The postcopulatory choice hypothesis predicts that females manipulate the retention and use of male sperm (Dickinson 1997). If it is postcopulatory then females remate quicker when the quality of a previous male is low. This occurs in cases when early female choices cannot be made so that ejaculates may still be actively selected or rejected in different quantities. Tests

are centered around measuring the effects of both males and the females on the ejaculate distribution patterns.

Millipedes generally have highly polygynandrous mating systems that are very complex because males and females can mate multiply and prolong copulations to different degrees. The dynamics of mating and maintained genital contact have been thoroughly investigated in the spirostreptidan millipedes, and particularly *Alloporus uncinatus* (Telford & Dangerfield 1990a, b, 1993, 1996, Barnett 1991, 1997, Barnett *et al.* 1991, 1993, 1995, Barnett & Telford 1994). Males control the duration of copulation and maintain genital contact with their females in relation to the intensity of sperm competition (Telford & Dangerfield 1990a). This supports the adaptive mate guarding hypothesis, yet the (passive) role of the female that endures copulation is vague. The possible role of the female that endures and controls copulation duration, which ultimately is a limiting factor for males, has been neglected for ages despite the possession of a sperm-storage organ being a primary predisposition to high variance in sperm competition (Dewsbury 1982; Birkhead & Moller 1992).

The longest copulator known in a spirobolidan millipede genus, *Centrobolus inscriptus*, provides the opportunity to test hypotheses on: (1) sexual conflict over copulation duration. The mating success of males depends significantly on that relative to their partner (Adams & Greenwood 1987, Bostos *et al.* 1996, Bourne 1993, Fairbairn 1990). When males have to overcome female resistance to mate, factors such as sexual size dimorphism can influence the outcome of the struggle (Arnqvist 1992, Rowe 1992, Weisenberg & Fairbairn 1994); (2) sequential mating and the remating interval. The function of repeated mating is examined by studying causes of variability in the rate of remating, assuming that the same factors causing the evolution and maintenance of multiple mating are responsible for variability (Brown

1997). Early remating has been proposed to be due to the storage of an unusually low number of sperm from the previous mating (Gromko *et al.* 1984; Newport & Gromko 1984). Sperm precedence and sperm movement can change with the remating intervals (Suzuki *et al.* 1996). Remating may also function as a mechanism of either precopulatory mate choice or relative postcopulatory mate choice between sequential matings (Brown 1997); (3) self-sperm displacement. Self-sperm displacement may have a role in flushing out rival sperm from the temporary sperm stores. In the spirostreptidans instantaneous insemination is followed by a sharp exponential decline in ejaculate volume (Barnett & Telford 1994); (4) sperm priority patterns of different males. Ejaculate volumes can be used to determine sperm usage patterns (Birkhead & Petrie 1995, Bishop 1996). Generally there are three possible scenarios, namely first-male, shared and last-male precedence (Dewsbury & Baumgardner 1981, Harshman & Prout 1994).

Materials and methods

Animal collection and maintenance

Millipedes were hand collected 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 ($\pm$ 5cm deep) before the mating experiments commenced.

Male radioisotope labelling, female spermathecae removal, and ejaculate detection

Ejaculate priority patterns were quantified by calculating V_2 - a volumetric indicator and possible analogue of P_2 - the proportion of offspring sired by the second male to mate with a

twice-mated female (Boorman & Parker 1976). The reason for selecting this measure was because *C. inscriptus* would not oviposit under laboratory conditions and so offspring counts could not be made. The experimental protocol was based on a strict *a priori* approach with the ejaculate being the unit for consideration (Dewsbury 1982). The radioisotope labelling technique allowed two types of ejaculates to be discerned (North & Halt 1968; and see Barnett *et al.* 1995 for its application in millipedes), A Hamilton syringe was used to inject 50 μ l aliquots of tritiated [*methyl*- ^{3} H] thymidine (85 Ci / 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 0.1ml concentrated HCl to promote rapid tissue homogenisation (Barnett & Telford 1994). Acid was neutralised with 0.1ml 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. The weakness of this method was that it did not take into account variation in sperm production, sperm quality, sperm motility within the female, intercellular sperm-egg interactions and intra-cellular sperm-egg interactions. These were all assumed to be negligible.

Mating experiments

Before being placed into mating arenas (glass aquaria 30 X 22 X 22 cm), individual animals were marked on the posterior segments with coloured correction fluid. This allowed every individuals mating history to be followed. Approximately five minutes after they had established a copula position, pairs were removed from the mating arena and placed into plastic beakers (13cm diameter) where the copulation duration was recorded. This removal was necessary to eliminate the effects of male-male competition on intersexual conflict.

Radiolabelled males were used in artificially-terminated matings to elucidate the timing of insemination (*sensu* Barnett & Telford 1994 in *A. uncinatus*). Copula pairs were separated after different time intervals within the first 30 minutes of copula. Double mating sequences were performed to test for mating order and interval effects. Mating order was controlled by mating a female first with a labelled male followed by an unlabelled male, and then *vice versa* (L-UL versus UL-L). Females were given the opportunity for a second matings either immediately after the first (0-hour delay) or approximately 24 hours later (24-hour delay). As controls, females that had single matings with labelled males were dissected immediately or after 24 hours (L(0) versus L(24)). Females that had single matings with unlabelled males were dissected in a similar manner to control for background radiation.

Morphometric data

Measurements of body mass (accurate to 0.01 g), body length (mm), and dorsal tergite width (mm) were taken for males and females at the end of mating, in order to test for size-dependent ejaculate contribution by males and size-dependent ejaculate storage by females. Body sizes were perceived as volumes as calculated using the formula for the cylindrical *bauplan* (Chapter 2), from which sexual size dimorphism (SSD) was indexed.

Statistical analysis

Normally distributed data were analysed with Pearson's correlation (r) and t-tests (t) while Spearman's rank order correlation (r_s) and two-tailed Mann-Whitney U-tests (Z) were selected as non-parametric analogues with Wilcoxon tests (T) used on any matched pairs. The Kruskal Wallis 1-way Anova (H) was used to test for differences in dpm data prior to running the Mann-Whitney U-tests

Results

Morphometrics

Males and females differed significantly from each other in mass (Wilcoxon matched pairs test: $T = 554$, $n = 88$, $P = 0.00$), length ($T = 485$, $n = 88$, $P = 0.00$), and width ($T = 2.5$, $n = 88$, $P = 0.00$). The pairing of individuals within the mating arenas seemed to be random, therefore no relationships between the relative sizes of males and females within mating pairs was expected.

Copulation duration

Copulation in *C. inscriptus* lasts for 173 ± 41 min (Mean $\pm$ sd, $n = 46$). First (174 ± 35 min) and second (173 ± 47 min.) copulation durations of females were not significantly different ($T = 507$, $n = 46$, $P = 0.91$) and were in no way related to each other ($r_s = -0.06$, $n = 46$, $P = 0.69$). The remating interval was not impacted by the first copulation duration ($r_s = 0.07$, $n = 46$, $P = 0.64$) but was negatively related to the second copulation duration ($r_s = -0.31$, $n = 46$, $P = 0.04$), so that when a female endured a long interval the following copulation was shorter (Figure 7.1). This result can be represented another way to show the differences between the first and second copulation durations of each female (Figure 7.2).

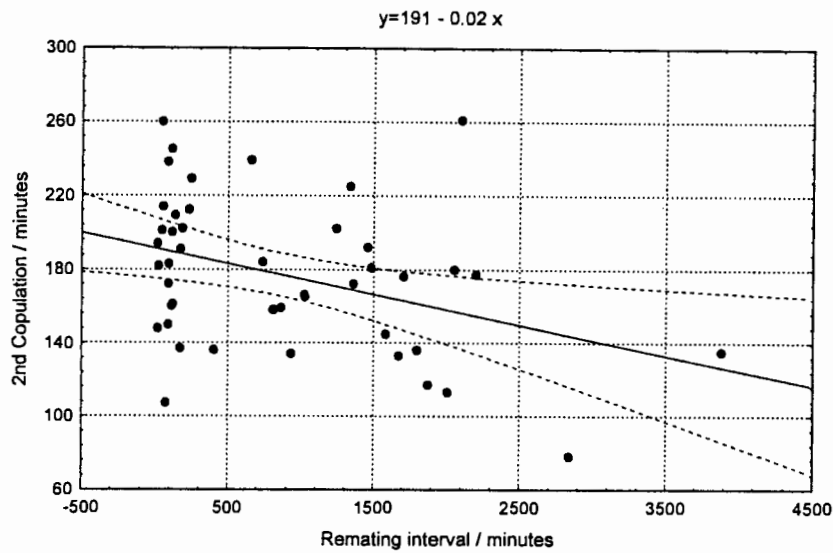


Figure 7.1 The change in the second copulation durations of female forest millipedes *Centrobolus inscriptus* that mated after progressively longer intervals renders a slope possibly indicative of sexual control. $r_s = -0.31$, $n = 46$, $P = 0.04$

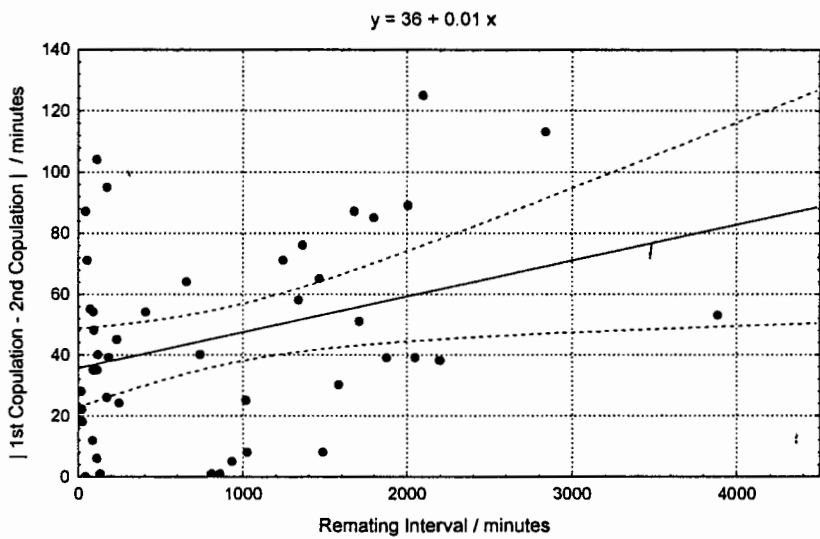


Figure 7.2 The absolute difference in durations between successive copulations performed by individual female forest millipedes *Centrobolus inscriptus* that had different remating intervals. $r = 0.33$, $n = 46$, $P < 0.05$

Male and female allometries

Although female girth was positively related to copulation duration (chapter 6), the absence of this relationship in the first copulation durations of double matings ($r_s = 0.19$, $n = 46$, $P = 0.22$) means that a type II error is possible. Female girth was again positively correlated to the second copulation duration ($r_s = 0.31$, $n = 46$, $P = 0.04$), suggesting that this is no artefact and should be discussed further.

Male length was positively related to the duration of the first mating ($r_s = 0.30$, $n = 46$, $P = 0.04$) but negatively related to the duration of the second mating ($r_s = -0.35$, $n = 46$, $P = 0.02$). This is interesting when considering the lower variance of male lengths ($CV = 4.0$) relative to female lengths ($CV = 5.7$) and that lower variance may indicate directional sexual selection (Chapter 2). The absence of a relationship between male length and width ($r_s = 0.13$, $n = 46$, $P = 0.39$) supports this statement because if both they were related their increase would be an effect of maximizing the volume of the cylindrical bauplan rather than length *per se* (see Chapter 2). In order to resolve the relationships of copulation duration with both male and female morphometric parameters sexual size dimorphism was calculated.

Sexual size dimorphism

The first copulation duration was not significantly dependent on the sexual size dimorphism within copulating pairs (Figure 7.3, $r = 0.2$, $n = 46$, $P > 0.05$), but the second copulation durations was ($r = 0.41$, $n = 46$, $P = 0.004$). The absolute differences between first and second copulations (Figure 7.2), together with these results render slopes that are all potential indicators of the degree of sexual conflict.

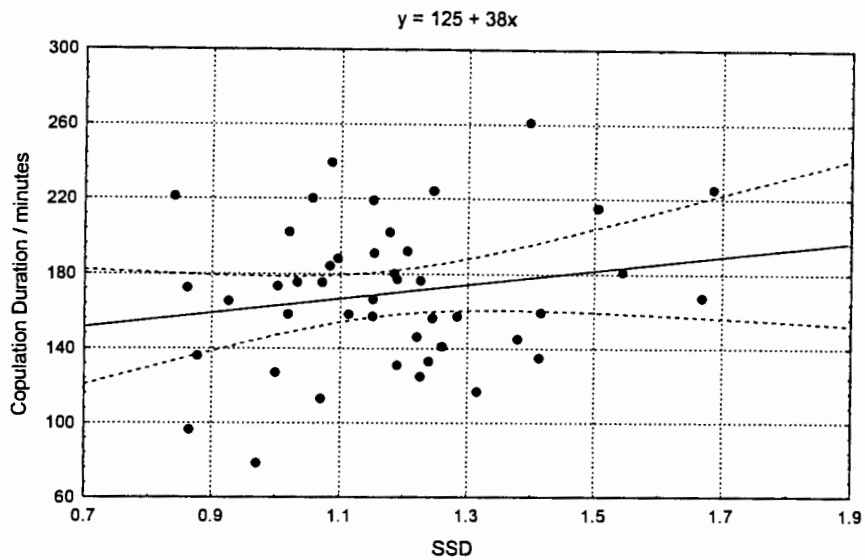


Figure 7.3 The first of two copulation durations by forest millipedes *Centrobolus inscriptus* where mated pairs varied in sexual size dimorphism (SSD). $r = 0.2$, $n = 46$, $P > 0.05$

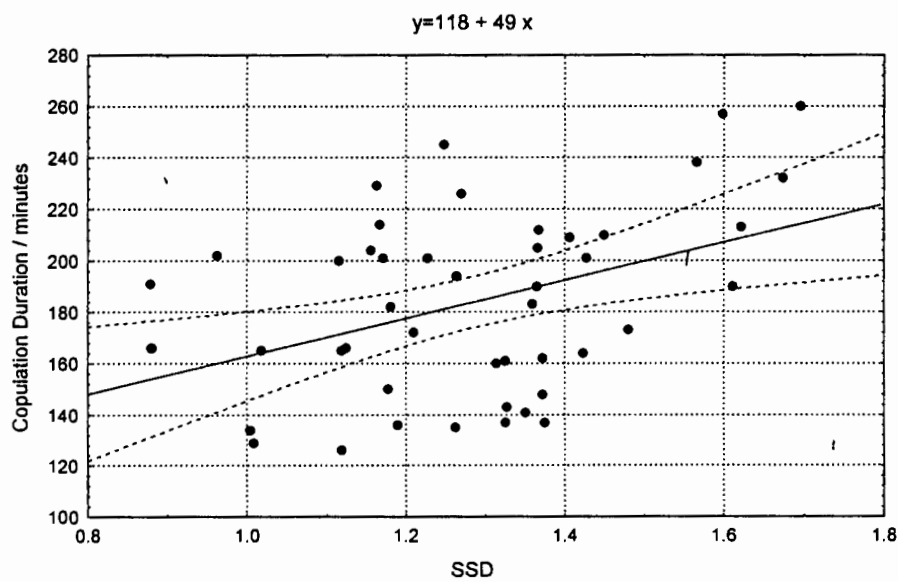


Figure 7.4 The second copulation durations of forest millipedes *Centrobolus inscriptus* where mated pairs had different degrees of sexual size dimorphism (SSD). $r = 0.41$, $n = 46$, $P = 0.004$.

Labelled ejaculates

There was no significant difference between the copulation durations of females mating with labelled and unlabelled males ($t = 0.38$, $df = 28$, $P = 0.70$) so it is safe to assume that the radiolabel did not advert male mating behaviour in any way that would change the results of copulation durations. No left-right differences occurred in any of the mating sequences ($T = 711.5$, $n = 59$, $P = 0.19$) indicating that equal volumes of ejaculate were deposited in the left and right vulvae of females and so dpm values could be added without any confounding effects, e.g. due to fluctuating asymmetry.

Artificially-terminated matings

Ejaculate volumes inseminated did not correlate significantly with the manipulated copulation durations ($r = -0.35$, $df = 8$, $P = 0.35$). The first few data points indicate incredibly high volumes deposited within the first five minutes interspersed with incredibly low volumes (Figure 8.5). Although this could be variable "instantaneous-insemination" it is not necessarily and will be considered "early-insemination" during which time the male loads and seats the gonopods. Male body mass was not related to ejaculate volume ($r = 0.54$, $df = 8$, $p = 0.13$) but it was closely related to copulation duration ($r = -0.64$, $df = 8$, $P = 0.06$).

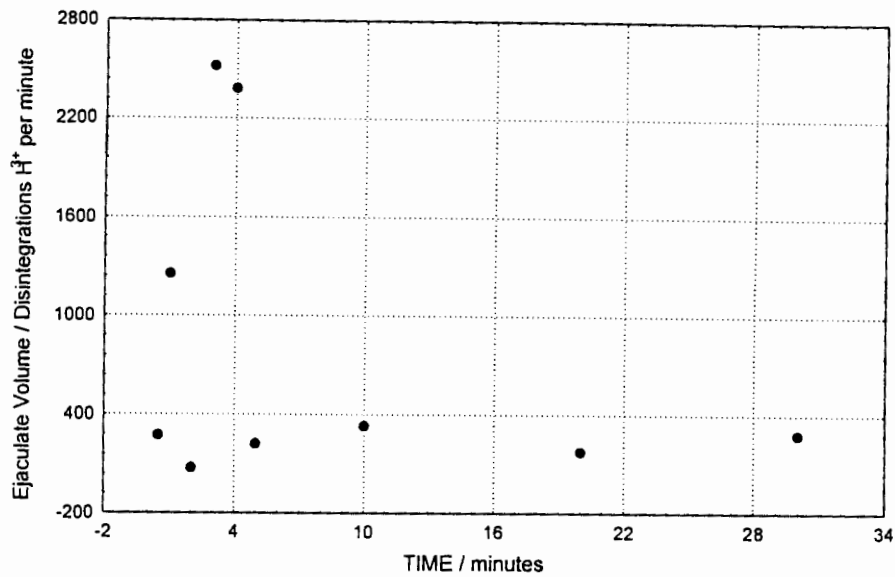


Figure 7.5 The ejaculate volumes inseminated by radiolabelled males of *Centrobolus inscriptus* when matings were artificially terminated at different stages.

Single and double mating sequences

There were significant differences between total dpm values measured after the mating series (Figure 7.6: $H = 30.67$, $df = 5$, $P = 0.00001$). The significantly higher dpm values of the first male relative to those of the second are obvious when there is no delay between double matings ($L-UL(0) > UL-L(0)$: $Z = 3.81$, $n = 12,12$, $P = 0.0001$). The immediate impact of the second male on the females sperm stores was not significant ($L(0) \sim L-UL(0)$: $Z = 0.84$, $n = 6,12$, $P = 0.40$), and the ejaculate volumes remain significantly higher than those introduced by the second male ($L(0) > UL-L(0)$: $Z = 3.37$, $n = 6,12$, $P = 0.0007$). These results indicate that a second mating does not affect the ejaculate of the first male, however its presence has an effect on that of the second male ($V_2 = 0.21$).

When there is a delay of 24 hours between successive matings, volumetric precedence reverses and the dpm values of the second male are significantly higher ($UL-L(24) > L-UL(24)$): $Z = -2.59$, $n = 8, 14$, $P = 0.01$). The effect of the delay upon the relative dpm values of first and second males ($V_2 = 0.77$) may be due to a male or a female mediated process, or both.

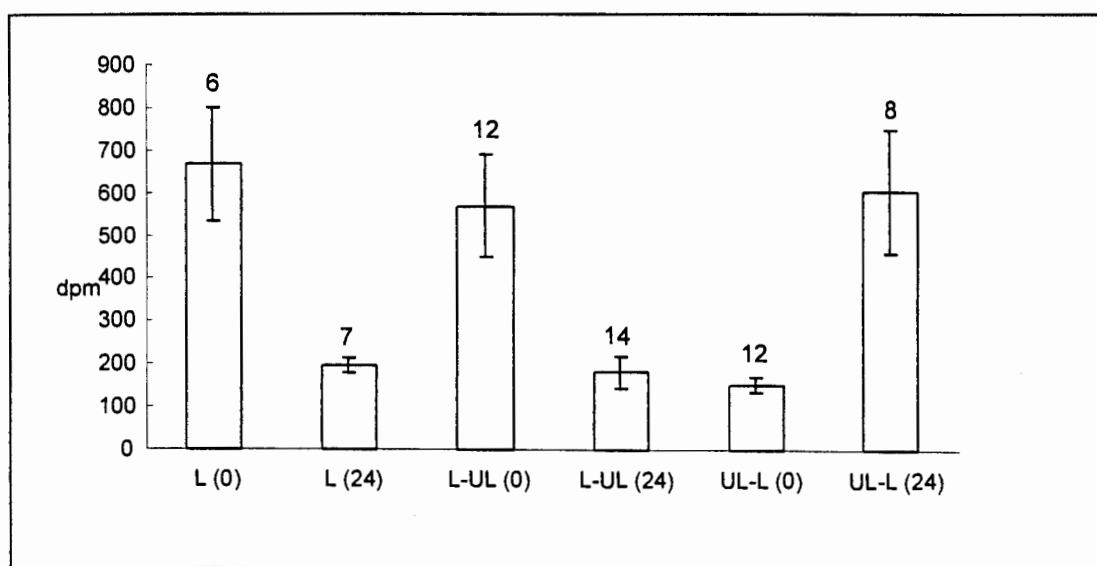


Figure 7.6 Ejaculate volumes (mean disintegrations H^{3+} per minute $\pm$ SE) obtained from the spermathecae of *Centrobolus inscriptus* females engaged in single and double mating experiments where (0) and (24) represent the period (hours) before dissection and the remating intervals, respectively. L - H^{3+} labelled male, UL - unlabelled male

The absence of any differences in dpm values between single and double matings with controlled intervals ($L(0) \sim L-UL(0)$: Z above; $L(24) \sim L-UL(24)$: $Z=0.58$, $n = 7, 8$, $P = 0.56$) rejects the male ejaculate removal hypothesis. The significant drop in dpm over the 24 hr period postcopulation ($L(0) > L(24)$: $Z = 3.00$, $n = 6, 7$, $P = 0.003$) indicates that there is female mediation of the stored sperm. Hence, the female process hypothesis is accepted over the male process hypothesis ($L-UL(0) > L(24)$: $Z = -3.13$, $n = 7, 12$, $P = 0.002$).

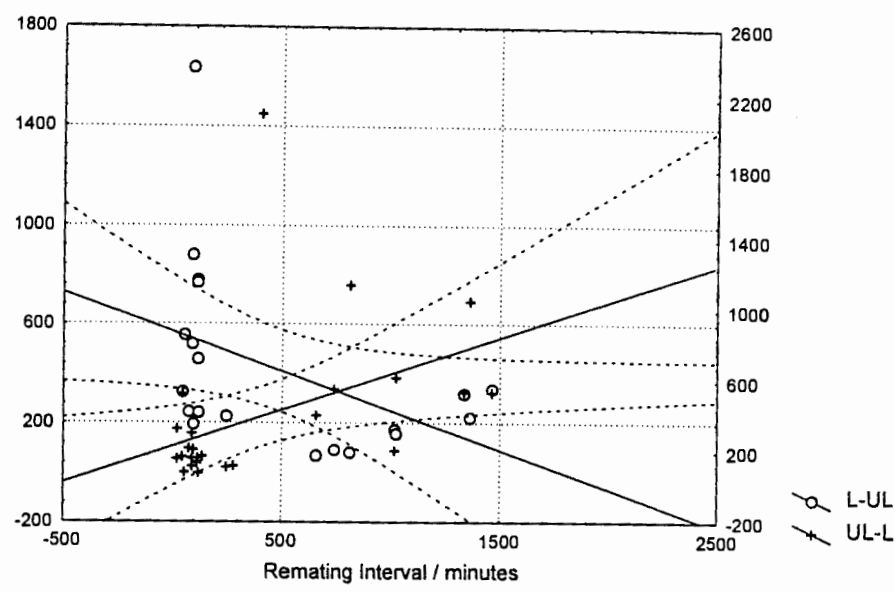


Figure 7.7 The change in ejaculate volumes (disintegrations H^{3+} per minute) originally deposited by the first male (L-UL) and those deposited by the second male (UL-L) after different remating intervals are indicative of volumetric ejaculate priorities.

Sperm displacement

Sperm displacement was negatively related to copulation duration when there was a 24-hr remating interval (L-UL(24) dpm vs copulation duration male 2: $r_s = -0.88$, $P = 0.004$, $n = 8$), indicating that self-sperm displacement is higher than rival sperm displacement. This relationship falls away when the females mate in quick succession (L-UL (0) dpm vs copulation duration male 2: $r_s = -0.10$, $P = 0.75$, $n = 12$).

Discussion

The high variation in prolonged copulations of *C. inscriptus* stresses how the degree of control by either sex varies from one copulation to the next. More explicitly, the results show that there is a conflict of interests over controlling copulation duration. Male strategies are affected by the remating interval and the volume of last male ejaculate still present in the female vulvae. Female strategies that counter male-male competition are based on sperm dumping postcopulation. It is debatable whether females can directly counter male control by parasitizing mate guarding behaviour.

Male strategies

Males prolong the duration of copulation as a post-insemination guarding tactic when sperm competition intensifies. The high levels of ejaculate recorded early in copulation resemble the spirostreptid millipede pattern of "immediate insemination" followed by an exponential decline indicating self-sperm displacement during the adaptive guarding phase (Barnett & Telford 1994, Barnett 1997). The large variability in the ejaculate volumes which are inseminated early makes *C. inscriptus* different from the patterns described for other millipede taxa. The multiple "insertion-withdrawal" of gonopods may be what influences sperm leakage and flowback in *Centrobolus* (M. Barnett *pers. comm.*). This "early-insemination" and the absence of a step-wise increasing further confirms that prolonged copulations in millipedes do not function to maximize ejaculate transfer (see also Barnett & Telford 1994, Barnett 1997). In the water strider *Aquarius remigis*, where complete sperm transfer takes approximately 20 minutes (Rubenstein 1989), there are other reasons why males remain *in copula* with inserted genitalia for many hours (Wilcox 1984, Clark 1988, Rubenstein 1989, Sih *et al.* 1990, Fairbairn 1990).

With some of the ejaculates inseminated being over twice the volume of that contained by twice mated females it is possible that self-sperm displacement functions to maximize total sperm displacement. This is indeed what the double mating sequences prove. With a 24-hour interval sperm displacement is almost paradoxical but when one considers that this does not happen when females mate in quick succession, the self-sperm displacement strategy becomes clear. Self-sperm displacement is less effective after the mating interval because rival sperm has already been removed from the temporary sperm stores by the female. In combination these displacement activities will result in sperm competition by mixing and dilution, hence there is "raffle" competition (Birkhead & Moller 1992). In these cases where copulations are prolonged beyond the time necessary for insemination, males can guard females by acting as live mating plugs and also counter sperm competition by displacing previous male ejaculates (Parker 1970, Sillen-Tullberg 1981, Thornhill & Alcock 1983).

Exactly the same pattern of results were found in the spirostreptidan millipede *A. uncinatus* (Barnett *et al.* 1995), however the genital mechanics are very different.

A huge number of male inactivating mechanisms and cryptic female mechanisms may occur between ejaculate storage and final paternity assurance (i.e. fertilization), and between female sperm usage and offspring production respectively (Eberhard 1996). Lowering the ejaculate volume, and presumably also the number of sperm belonging to the previous male is obviously reproductively advantageous for males that obtain temporary sperm precedence. The main implications of sequential mating is that temporary sperm precedence increases as a function of the refractory period, shifting from 21% with no remating interval to 77% after 24 hours. Elongate males benefit by terminating copulations earlier because they "know" that they will achieve high precedence. If the fraction of ejaculate stored by the female were the only factor responsible for paternity then permanent sperm precedence, P_2 (the proportion of

offspring sired by the second male to mate with a twice mated female *sensu* Boorman & Parker (1976), would be indicated by temporary sperm precedence, V_2 (the proportion of ejaculate deposited by the second male into the storage organ of a twice mated female). The increase in temporary sperm precedence with the decline in previous ejaculate volumes during the 24 hour remating interval needs to be addressed from a female perspective.

Female strategies

On average 71% of male ejaculate gets emitted by the female within 24 hours of terminating copulation. The egg simulation hypothesis, predicts that male insemination activity and gonopod movements cause tensions on female bursa muscles that results in sperm ejection, is partly corroborated. However, the sperm of the previous male is ejected from the receptaculum or spermathecae postcopulation and not early in copulation as is the case in the 'higher' julids (Tadler 1996a). Artificial ejection and sperm-secretions have been found on the openings of the receptacula of female millipedes that were fixed without having remated (Blower 1985, Tadler 1996b). The evidence for sperm dumping postcopulation in *C. inscriptus* eliminates the previous fixation artefact of sperm secretion and makes male stimulation a possible cause.

In *Drosophila melanogaster*, first male sperm lost from storage when females remate within 6 h was taken as indirect evidence for sperm displacement (Scott & Richmond 1990). This loss, or displacement, of first male ejaculate may be a "previous male effect", e.g. induction by seminal fluid components (Scott & Williams 1993). A lack of consistency in the proportion of different male ejaculates that were dumped may have reproductive consequences for males but are ultimately under female control. The remating interval may further be determined by female state, which could itself influence the patterns of ejaculate

priority (Suzuki *et al.* 1996). Females benefit through control of sperm usage during the remating interval in several ways. Female-mediated behaviours, such as sperm dumping, are important as they present females with an opportunity to selectively absorb sperm in accordance with male quality and so make a 'cryptic choice' (Eberhard 1996).

One mechanism of cryptic female choice is to "sometimes discard sperm of previous males." (Eberhard 1996). Postcopulatory choice is possible by a discharging process (Parker 1970, Rodriguez 1994), however, choosing mates sequentially by relative postcopulatory comparisons often requires the ability to "remember" previously visited males for mating comparisons (Bakker & Millinski 1991, Real 1990) that would require a neuronal basis (Jacobs 1996). This questions the foundations on which female choice in millipedes even if it is postcopulatory. Still, if a postcopulatory choice were being made, the biased use of stored sperm would be very difficult to prove empirically. Females may retain larger volumes of the second males ejaculate in order to enhance muscular movements and dump the first males ejaculate from the ampullae.

The volume of female sperm storage organs relative to male ejaculate size is a potentially important factor in cryptic female choice since it could affect sperm precedence. Parker (1970) argued that males, since they produce cheaper gametes, are strongly selected to fill up the female with more than enough sperm to fulfil all of her anticipated needs for fertilization. In general, the volumes of sperm transferred are expected to be similar to the capacities of female sperm storage organs (Eberhard 1985: 102). The difference between the total ejaculate volume inseminated and that remaining to be stored implies that even if there is a volumetric constraint imposed by the female sperm storage organ, self-sperm displacement is still effective. With capacity limits for female sperm storage, males will

suffer greater costs through self-sperm displacement yet also be more efficient in displacement under “raffle” competition. This has important implications in the outcome of sexual conflicts even though the males remain the static sex by indiscriminate sperm displacement. Factors that affect sperm emission from the female post-copulation are unclear but the greater the volume of sperm dumped does not necessarily imply ejaculate rejection but that higher storage capacity is available for the subsequent male.

How are sexual conflicts over copulation duration resolved?

The larger animals are invariably the victors. The most nagging question is why are second copulations shorter than the first if larger females tend to endure longer copulations yet not retain significantly greater volumes of sperm? One answer could be that second copulations are male controlled and terminated earlier due to lower SSD. In mating pairs characterized by low SSD the male can assume more physical control over the female than in situations characterized by high SSD where the female is significantly larger. In matters of physical combat, the relative sizes of the combatants are of primary importance (Wallace 1987).

The answer to the second part of the question, why larger ejaculate volumes are not retained, is alluded to by two factors. Firstly, the females have a temporary as well as a permanent sperm store. Secondly, the higher variance in second male ejaculates after longer remating intervals. The effects of sperm dumping and second male sperm displacement are masking. The way to test between these alternatives is to calculate the trade-off between male self-rival sperm displacement, and female dumping - male displacement by employing two different types of radiolabels. Entire ejaculates do not enter and remain in the female sperm stores, due to the combined loss through rival-/self-sperm displacement and female sperm dumping, not all sperm are equally likely to be used for fertilization. A prolonged copulation

may be costly to a female deprived of a mating interval during which sperm usage, fertilization and oviposition, can be exercised irrespective of 'cryptic female choice'.

Variation in copulation duration has been attributed to the sequence number of the copulation for the male on a day (Michiels 1992). The variability in the interval between successive copulations, like that of the actual copulation duration, may have evolved through male or female controlled processes. The prolonged remating intervals are unlikely to be the result of induced unreceptivity whereby the first male delays a female remating because second males have the higher potential volumetric sperm priority after longer intervals. The operational sex ratio (OSR) is another factor responsible for natural variance in remating intervals. It is proposed that a male-biased OSR will cause a reduction in the remating interval.

In order to make predictions about sperm precedence (P_2 *sensu* Boorman & Parker 1976) from these results, and test the proposed model, further investigations will need to consider how the sperm from different males are used in proportion to their volumetric representation in the sperm stores. There are a plethora of factors that affect sperm priority patterns and these can easily disappear after manipulating a single factor, e.g. sperm precedence breaks down after a third mating (Zeh & Zeh 1994). Areas in which the current results have succeeded most are in showing differential sexual conflict over copulation duration based upon sexual size dimorphism. A general conclusion is that the mechanisms of sperm displacement and sperm dumping are incompatible and that the proportion of different male ejaculates that were dumped represents female variation that can have reproductive consequences for males.

Polygynandrous mating systems in sympatric millipedes: skewed sex ratios, “faster” sex and a conflict of interests

The finding that genitalic diversity and complexity evolves in response to a conflict of interests between males and females has generated five predictions (Alexander *et al.* 1997): (1) Females mate multiply, (2) sperm precedence is not complete, (3) fertilization is separate from insemination, (4) males cannot entirely sequester females following insemination, and (5) males do not participate in parental care. Sperm precedence has been quantified as incomplete in *C. inscriptus* (previous chapter), fertilization can be decoupled from insemination by female control of sperm movements (Chapter 4), and male millipedes do not participate in parental care. This leaves predictions (1) and (4) open for testing.

When females mate multiply, the relative number of sexually receptive males and females available to mate directly affects the differences in mate competition between the sexes (Emlen & Oring 1977). Otherwise known as the operational sex ratio (OSR), this is the immediate factor determining which sex will compete most intensely for mates (Clutton-Brock & Parker 1992, Kvarnemo & Ahnesjö 1996). Male-male competition is predicted to intensify with male biases and female-female competition with female biases. Potential reproductive rates (PRR) of individuals follow this shift in the OSR (Kvarnemo *et al.* 1995) so that a “faster” sex always emerges in the population. The faster sex possesses a suite of behavioural adaptations that augment mating success in a competitive

environment. These behaviours are predicted to exacerbate a conflict of interests between the sexes.

Several species of millipede are known to coexist in a given habitat (Dangerfield & Telford 1993), but it is unknown whether the OSR is linked to a conflict of interests. Having two species of millipedes, *Centrobolus inscriptus* (Attems 1928) and *C. anulatus* (Attems 1934), in sympatry provides the opportunity to test for differences in the degree that females mate multiply and the abilities of males to sequester females following insemination. After drawing on the similarities and differences that have already been found between the two species, consideration will be given to (1) how the OSR can be modified by dispersal (Kvarnemo & Ahnesjö 1996, Reynolds 1996). The differences in the relative abundance and dispersal of males will determine female remating frequencies; (2) why males become the "faster" sex in the population (Clutton-Brock & Parker 1992); (3) the degree of "elaboration" or genital complexity and other morphological structures of the faster sex (*sensu* Eberhard 1985, 1996). Generally, the morphology and behaviour of the faster sex is expected to have evolved through higher intrasexual selection and more costly conflicts of interest between males and females.

Materials and Methods

Field data

The data were collected during the rainy season because in southern Africa millipede surface activity is strongly seasonal and related to feeding and reproduction (Dangerfield & Telford 1991, Dangerfield et al. 1993) and this is also when population densities peak (Dangerfield & Telford 1989). The two species of millipedes were sampled in their indigenous tropical coastal forest habitat at Twin Streams Farm, Mtunzini, South Africa (28°55'S; 31°45'E). It is within this part of typical coastal forest belt (Acocks 1975) that *C.*

anulatus and *C. inscriptus* are in geographical sympatry. An area of forest with continuous tree canopy cover was delimited and all sampling occurred within those bounds.

In the first season two temporally separate sampling efforts were made and the OSR was measured early in the season (OSR_{early}) and OSR_{late}). In the following season a single sampling effort involved hand collecting all individual millipedes on the ground and in shrubs and trees separately, with the former measured as OSR_{ground} and the latter as OSR_{tree} (>30cm but <3m above ground surface; see also van Aarde *et al.* 1996). The two species were morphologically separated based upon the presence of yellow flashes, red legs and red heads (*C. inscriptus*) vs. discrete red-black annulations without yellow flashes, black legs and black heads (*C. anulatus*). The number of mating pairs found *in copula* was recorded and every individual was immediately sexed based on the presence of gonopods in the males. Over a period of three full days the total numbers of each species collected were used to calculate the relative abundance of each species within the forest patch.

Relative abundance, OSR and copulation frequency

The total number of individuals collected were taken as a measure of relative abundance so that sample sizes (n) are biologically meaningful. The conventional measure of OSR was calculated as a percentile of males / males + females. Both measures were partitioned into the ground and tree substrata accordingly. The copulation frequencies were calculated as the number of copulating pairs divided by the population sample.

Pilot mating experiments

Even after having been separated by sex, males of *C. inscriptus* proceeded to attempt to mate indiscriminately with other males. Although cross-matings had never been observed

and no hybrids exist in the wild, cross-mating attempts of *C. inscriptus* males with *C. anulatus* females were induced after individuals of both sexes and both species were placed together during the collecting period.

Laboratory mating experiments

Live specimens were transported to the laboratory, where unisex groups were housed in plastic containers containing moist vermiculite (± 5 cm deep), 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*. In the first trial ten *C. inscriptus* males were placed in a glass mating arena (30 X 22 X 22 cm) together with ten male and ten female *C. anulatus*. Copulation durations were recorded by isolating the mating pairs using plastic beakers (13cm diameter) as they formed. In the second trial only conspecific matings of *C. inscriptus* and *C. anulatus* were performed in the same manner as the interspecific matings with ten individuals of each sex so that the OSR was at equality.

The radio-isotope labelling technique (see previous chapter) was used to determine whether sperm was transferred during interspecific matings. After 24 hours when the isotope had distributed in the haemocoel the interspecific mating experiments were performed. Females were killed in ethyl-acetate jars immediately afterwards, their spermathecae dissected, and the volume of labelled ejaculate quantified in disintegrations per minute (dpm) (see previous chapter for technique).

Results

Interspecific differences

C. inscriptus and *C. anulatus* are similar in sexual size dimorphism ($SSD = 1.2$); in both species the females are larger. The species differ in (1) the grasping structures on the legs of males. *C. inscriptus* has larger tarsal pads than *C. anulatus* (Chapter 2); (2) male accessory genitalia. The gonopods of *C. inscriptus* have a 20% more prominent sternite and spines that are double the length of those found in *C. anulatus* (see Table 3.1). It is difficult to say whether the one is more 'complex' than the other, but they are certainly species-specific; (3) *in copula* position. *C. inscriptus* is a coiling copulator and *C. anulatus* tends to be a parallel copulator. Also, the former species has a more pronounced sexual collar that is inflated during copulation; (4) average copulation duration. *C. inscriptus* copulations are over twice as long as *C. anulatus* (Chapter 6).

Since the swarms of both species were pure ($> 95\%$ adults) this allowed the sampled sex ratio to provide a good indication of the OSR. The few data indicate that the OSR is unstable and exhibit spatial and temporal changes

Temporal distribution and OSR

OSR_{early} were higher than OSR_{late} and densities were lower later in the season for both species (Table 8.1). In *C. inscriptus* the OSR demonstrates a seasonal shift from male to female bias. The absence of significant biases in the OSR_{early} and OSR_{late} for *C. anulatus* is prone to a Type II error. The OSR_{early} is best considered near male biased because it is a higher percentile than the same value for *C. Inscriptus*. However, the OSR_{late} is accepted to equalize.

Table 8.1 Operational Sex Ratios (% = males / males + females) recorded early (OSR_{early}) and two months later (OSR_{late}) in the mating season of two sympatric millipede populations, *Centrobolus inscriptus* (Attems 1928) and *C. anulatus* (Attems 1934). Note: abundance is indicated by the sample sizes designated in brackets (n).

Species	OSR_{early}	OSR_{late}	χ^2	P
<i>Centrobolus inscriptus</i>	60 % (800)	33 % (135)	32.00, 16.36	0.001*, 0.001*
<i>C. anulatus</i>	62 % (75)	57 % (46)	4.81, 0.78	NS, NS

* significant bias in the OSR relative to equality (goodness of fit tests: expected OSR = 50%).

Spatial distribution and OSR

The overall relative density of *C. inscriptus* over *C. anulatus* is almost ten to one (9.41 : 1) but there are several differences in the OSR between the two species (Table 8.2). In *C. inscriptus*, the OSR was not significantly male biased on the ground substrate ($\chi^2=0.8$, $P > 0.25$, $df = 1$) but was significantly male biased in the tree substrate ($\chi^2 = 25.73$, $P = 0.001$, $df = 1$); these combined to produce an overall male bias ($\chi^2 = 181.64$, $P < 0.001$, $df = 1$) in the population. In *C. anulatus*, the sex ratio was not significantly male biased ($\chi^2 = 62$, $P > 0.25$, $df = 1$).

Table 8.2 Operational sex ratios (% = males / females + males) on the ground (OSR_{ground}) and tree substrata (OSR_{trees}) inhabited by sympatric millipedes, *Centrobolus inscriptus* (Attems 1928) and *C. anulatus* (Attems 1934). Note: abundance indicated by sample sizes (n).

Species	OSR_{ground}	OSR_{trees}	OSR
<i>Centrobolus inscriptus</i>	55 % (101)	62 % * (445)	65 % * (546)
<i>C. anulatus</i>	-	55 % (58)	55 % (58)

- denotes the species absence from the stratum. * denotes significant bias in the OSR relative to equal male : female frequencies (expected OSR = 50 %); tests were made by goodness of fit (χ^2).

Copulating pairs

The mating frequency of *C. inscriptus* was calculated as 12.09% pairs in copula, partitioned as 30.59% being on the ground and 69.41 % in the trees, compared to 3% for *C. anulatus* that were only present up in the trees. Three pairs of *C. inscriptus* were found to be accompanied by a second male ('triplets') and another mating group consisted of two pairs *in copula* accompanied by another male ('clusters').

Interspecific mating experiments

In the first trial copula pairs formed within 20 minutes of introducing *C. inscriptus* and *C. anulatus* males to *C. anulatus* females. Eight of the matings were heterospecific (i.e. *C. inscriptus* X *C. anulatus*) with copulation durations that ranged from two to 27 minutes (12.4 ± 10.6 min). The last mating was between two conspecifics (*C. anulatus* X *C. anulatus*) that lasted 30 minutes. Another seven such matings were performed separately in order to calculate a mean copulation duration for this species (Table 8.3). In both con- and heterospecific matings genital contact was established with the male inserting the gonopods into the females vulvae and forming a sexual collar. In *C. inscriptus*, copulation durations were longer than those of *C. anulatus* (Two-tailed Mann-Whitney U-test: $Z = 2.56$, $n = 8,6$, $P = 0.01$; see Table 8.3: conspecific matings).

Interspecific matings revealed that radiolabelled ejaculates were transferred by *C. inscriptus* males and they were not significantly different in volume to those inseminated during conspecific matings (Mann-Whitney U-test: $Z = 1.23$, $n = 8,6$, $P = 0.22$) although there is large variability (Table 8.3). The absence of mate discrimination by *C. inscriptus* males sometimes led to homosexual mating attempts. Indiscriminate male mating

behaviour is so pronounced that attempts at heterofamilial mating with *Alloporus* sp. over three time their body size were made when contained together in small tanks (*pers. obs.*).

Table 8.3 The results of interspecific laboratory mating experiments performed between two naturally sympatric millipedes, *Centrobolus inscriptus* (Attems 1928) and *C. anulatus* (Attems 1934), with information on the copulation duration and ejaculate volumes inseminated (mean $\pm$ SE) in matings with both conspecific and heterospecific partners.

Mating behaviour	Mating partners (male X female)	Copulation duration (min)	n	Ejaculate volume (dpm)
conspecific*	<i>C. inscriptus</i> X <i>C. inscriptus</i>	145.5 $\pm$ 29.6	6	668 $\pm$ 327
	<i>C. anulatus</i> X <i>C. anulatus</i>	39.4 $\pm$ 18.6	8	-
heterospecific	<i>C. inscriptus</i> X <i>C. anulatus</i>	12.4 $\pm$ 10.6	8	443 $\pm$ 235
	<i>C. anulatus</i> X <i>C. inscriptus</i>	0	0	0
homosexual	<i>C. inscriptus</i>	<5	8	-

A copulation duration of 0 indicates no mating attempt and 0 for no ejaculate transfer; - was not measured.

Discussion

The comparison between *C. inscriptus* and *C. anulatus* in sympatry indicates that spatial and temporal shifts in the OSR are responsible for differences in male-male competition. This competition is manifest by typically longer, coiled copulations and larger sexual collars. These behaviours, together with grasping structures and gonopods suggest that *C. inscriptus* has more intense conflicts of interest between males and females.

The temporal distribution of males and females

The tendency for the OSR to change through a breeding season may indicate that males and females emerge onto the local breeding grounds at different times relative to the onset of the rainy season. Finding a male biased OSR in the trees but not on the ground suggests that males of the more abundant species arrive in the trees prior to the females and compete heavily for mates. The decline in numbers as the season progresses happens at a point where individuals move downwards back into their underground refugia. The females deposit their eggs underground and undergo a moult, while the males may transform into an intercalary stage when the gonopods are absorbed (Hopkin & Read 1992). One of the behavioural adaptations known to augment mating success in a competitive environment with male biased OSR is earlier arrival on the breeding grounds in the sex with the potentially higher rate of reproduction (Reynolds *et al.* 1986). The OSR can also be mediated by the synchrony of female arrival (Grant *et al.* 1995). The pattern of male-male competition in the millipede *Alloporus uncinatus* (Attems 1928) is affected by both temporal and geographical variation in the OSR (Telford & Dangerfield 1990*a, b*, 1993). Temporal variance in sex ratios may occur within hours in some species of odonates (*Crocothemis erythraea*, *Orthetrum cancellatum*, Siva-Jothy 1985). When male biases arise in populations, prolonged copulation evolves to combat sperm competition.

The spatial distribution of males and females

The presence of *Centrobolus* millipedes up in the trees may seem perplexing as they are detritivores, a functional feeding group usually confined to the terrestrial and subterranean realms (Hopkin & Read 1992). This tree climbing behaviour was observed in the Sphaerotheriid pill millipedes *Sphaerotherium cinctellum* and *S. punctulatum* (Haacker & Fuchs 1972), and the pachybolids *Centrobolus fulgidus* and *C. sanguinipes* (van Aarde 1996), however no behavioural significance was mentioned. Three ideas have been forwarded to explain the frequent climbing behaviour observed in the adult spirostreptid *Alloporus uncinatus*, odontopypids *Chaleponcus limbatus* and *C. digitatus*, and several harpagophorid millipedes (Dangerfield & Telford 1996). These ideas bear relevance to the tree climbing behaviour in *C. inscriptus* and *C. anulatus*.

(1) In waterlogged conditions, particularly in riparian forests with clay rich soils, their movement and possibly feeding activity is impaired. *C. inscriptus* and *C. anulatus* are arboreal throughout the mating season, and the former species is simultaneously present on the ground and in the trees, so the effects of waterlogging on mating behaviour are rejected for *C. inscriptus*.

(2) Access to additional food sources such as microbial populations. All arboreal *Centrobolus* feed opportunistically on common lichen, flaking bark and senescing leaves (*pers. obs.*). For each species to show dietary specialization, eventually leading to niche separation, is not considered the driving force. This hypothesis requires collecting empirical evidence to demonstrate movement in response to food and higher arboreal feeding activity. A useful comparative approach is awaited with the katydids. At high food availability, the OSR nears twice as many males to females, while at low food availability there is a preponderance of males among sexually active animals (Gwynne 1990). For the

purposes of this study it will be assumed that in *Centrobolus* mating in the trees necessitates feeding.

(3) Millipedes climb trees to avoid mates (Telford & Dangerfield 1996). *Centrobolus* can be found copulating on terminal branchlets and within crevices between bark, sometimes hanging upside down *in copula* with only the female grasping (*pers. obs*). In the waterstrider *Aquarius remigis*, males tend to accumulate in areas with relatively few sites for females to escape (Rowe *et al.* 1994). 'Hot spots' are characterized by high density, a male-biased sex ratio and a high female mating frequency.

Does copulation duration trade-off with copulation frequency?

This question must be considered from both male and female perspectives because there is inevitably an intersexual conflict at some stage in the mating sequence (Alexander *et al.* 1997). In situations where males have control over females, it is in the females interests to avoid unnecessary matings, however, in situations where females parasitize or endure prolonged copulations they could reduce the mating frequency of males but still increase the variance in sperm competition. Comparing *C. inscriptus* copulation durations on the ground with those in the trees

This same question has been asked in a comparison between the sperm displacement mechanisms of two libellulid dragonflies with disparate copulation durations (Waage 1986). Differences in the location of pair forming and copulation, the frequency and duration of mating and oviposition, and the nature of their postcopulatory interactions were considered to reflect a trade-off between many short copulations with little sperm removal and longer, less frequent copulations with more complete sperm removal. The positive

association between copulation frequency and duration in both *C. inscriptus* and *C. anulatus* suggests that males are not faced with this trade-off.

Both copulation duration and frequencies are relatively lower in *C. anulatus*. This stasis in male mating behaviour is somewhat controversial because the females are more widely dispersed. Selection to mate guard has probably relaxed because sperm competition is less intense, so males maximize neither the time spent *in copula* nor mating frequencies. Perhaps tree climbing is an adaptive female mating strategy for avoiding unnecessary matings? As long as the PRR of males remain depressed, the PRR of the two sexes may be identical or even higher for females (Clutton-Brock & Parker 1992). Thus the lower conflict of interests.

The cross matings between the two millipedes *C. inscriptus* and *C. anulatus* lasted no longer than when female *C. anulatus* mated with conspecific males, suggesting that the control over copulation duration by either sex breaks down. This result is incomplete and requires further data. Reciprocal crosses among inbred lines of *Drosophila melanogaster* with different mating durations showed that the male was responsible for most of the variation (MacBean & Parsons 1966).

Why is the 'faster sex' indiscriminate?

Indiscriminate male mating in *C. inscriptus* indicates how much 'faster' the males are to respond to the changing OSR. High male copulation frequencies and long durations are therefore inextricably linked with increasing an individual's potential reproductive success. By comparison, the low mating frequency of *C. anulatus* males suggests that the females may even be the 'faster' sex. In most species it is advantageous for males to have a

very low threshold for mating attempts, in order to fertilise as many eggs as possible (Alcock 1993). Indiscriminate male mating behaviour has been reported in common toads *Bufo bufo*, Australian buprestid beetles, fruit flies *Drosophila pseudoobscura*, *D. persimilis*, *D. melanogaster* and *D. simulans*, (all cited in Alcock 1993), *D. biauraria* (Tomaru *et al.* 1995), and northern elephant seals *Mirounga angustirostris* (Deutsch *et al.* 1990), however this behaviour has never been related to copulation duration.

Comparative mating behaviour

Mechanical coupling and the transfer of average ejaculate volumes by *C. inscriptus* males during one way cross matings refutes both lock-and-key and genitalic recognition hypotheses for the evolution of male genitalia in *C. anulatus*. Interspecific mating behaviour is known to occur in the craspedosomatid millipedes *Craspedosoma transsilvanicum* and *C. alemannicum* (Tadler 1993). There are slight differences between the male genitalia and intermediary structural states can be identified, however, there are no differences between the female genitalia. *C. inscriptus* and *C. anulatus* are a similar case scenario however the *Craspedosoma* hybridize in their zone of sympatry. In both cases the reason for refuting the lock-and-key and genitalic recognition hypotheses for the evolution of elaborate genitalia is that they both imply no sperm transfer.

If it is biases in the OSR that are directly responsible for differences in mate competition between the two species then the OSR is also a determining factor of a conflict of interests between the sexes. The OSR may be considered as a major factor in determining conflicts of interests between males and females in a population for two reasons. (1) If the OSR is stratified within the habitat and shifts on a seasonal basis, then sperm competition and prolonged copulations are expected to increase with male biases that is beyond female

control, and (2) The degree of control over terminating copulation durations may result in males becoming the faster sex and being unable to entirely sequester females following insemination.

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**I. A cladistic classification of the class Diplopoda (after Enghoff 1984a)
indicating the position of the genus *Centrobolus***

CLASS Diplopoda

SUBTERCLASS Pencillata

ORDER Polyxenida

SUBCLASS Chilognatha

INFRAClass Pentazonia

ORDER Glomeridesmida

Sphaerotheriida

Glomerida

INFRAClass Helminthomorpha

SUBTERCLASS Colobognatha

ORDER Platydesmida

Siphonophorida

Polyzoniida

SUBTERCLASS Eugnatha

SUPERORDER Nematophora

ORDER Stemmiulida

Callipodida

Chordeumatida

SUPERORDER **Juliformia**

ORDER **Spirobolida**

FAMILY **Pachybolidae**

GENUS ***Centrobolus***

ORDER Spirostreptida

Julida

II. The descriptively valid species of *Centrobolus* Cook 1897 with their synonyms.

1. *C. luctuosus* (PETERS, 1855) (= *Spirobolus luctuosus* PETERS, 1855)
2. *C. litoralis* (KOCH, 1865) (= *Spirobolus litoralis* KOCH, 1865; *Spirobolus tessellatus* PORAT, 1872; *Chersastus erythroproctus* JEEKEL, 1956; *Chersastus elizabethae* SCHUBART, 1966)
3. *C. coriaceus* (PORAT, 1872)
4. *C. formosus* (PORAT, 1872) (= *Spirobolus formosus*)
5. *C. saussurii* (PORAT, 1872) (= *Spirobolus saussurii* PORAT, 1872; *Spirobolus falcatus* VOGES, 1878; *Spirobolus saussurei* ATTEMS, 1928; *Chersastus saussareii* JEEKEL, 1956; SCHUBART, 1966)
6. *C. strigosus* (PORAT, 1872) (= *Spirobolus strigosus* PORAT, 1872)
7. *C. pococki* (PORAT, 1893)
8. *C. digrammus* (POCOCK, 1893) (= *Spirobolus sabulosoides* PORAT, 1893; *Spirobolus digrammus* POCOCK, 1893; *Chersastus digrammus* SCHUBART, 1966; *Chersastus faciatus* ATTEMS, 1928)
9. *C. atrophus* (ATTEMS, 1928)
10. *C. inscriptus* (ATTEMS, 1928)
11. *C. ruber* (ATTEMS, 1928)
12. *C. silvanus* (ATTEMS, 1928)
13. *C. splendidus* (ATTEMS, 1928)
14. *C. anulatus* (ATTEMS, 1934) (= *Spirobolus elegans* PORAT, 1872; *Chersastus rubrofasciatus*, SCHUBART 1966)
15. *C. striolatus* (ATTEMS, 1934)
16. *C. vastus* (ATTEMS, 1934)
17. *C. angelicus* (JEEKEL, 1956)
18. *C. dubius* (SCHUBART, 1966)
19. *C. lawrenci* (SCHUBART, 1966)
20. *C. miniatomaculatus* (SCHUBART, 1966)
21. *C. promontorius* (SCHUBART, 1966)
22. *C. rubricollis* (SCHUBART, 1966)
23. *C. saginatus* (SCHUBART, 1966)
24. *C. sanguineomarginatus* (SCHUBART, 1966)
25. *C. titanophilus* (SCHUBART, 1966)
26. *C. albitarsus* (LAWRENCE, 1967)
27. *C. bifidus* (LAWRENCE, 1967)
28. *C. decoratus* (LAWRENCE, 1967)
29. *C. fulgidus* (LAWRENCE, 1967)
30. *C. immaculatus* (LAWRENCE, 1967)
31. *C. inyanganus* (LAWRENCE, 1967)
32. *C. lugubris* (LAWRENCE, 1967)
33. *C. pusillus* (LAWRENCE, 1967)
34. *C. richardi* (LAWRENCE 1967)
35. *C. rugulosus* (LAWRENCE, 1967)
36. *C. sanguinipes* (LAWRENCE, 1967)
37. *C. transvaalicus* (LAWRENCE, 1967)
38. *C. tricolor* (LAWRENCE, 1967)
39. *C. validus* (LAWRENCE, 1967)

III. The terminological abbreviations used to describe *Centrobolus* genitalic parts

Male genitalia	Female genitalia
Anterior gonopods / Coleopods (Hoffman 1971)	<i>v</i> vulva
<i>b</i> basal joint of telopodite (Attems 1928)	<i>bc</i> bursa copulatrix
<i>d</i> distal joint of telopodite (Attems 1928)	<i>o</i> oviduct
<i>v</i> sternite (Attems 1928)	<i>co</i> common oviduct
<i>k</i> distal lobe of telopodite (Attems 1928)	<i>op</i> operculum
	<i>at</i> apodemic tube
Posterior gonopods / Phallopods (Hoffman 1971)	<i>am</i> ampullae
<i>c</i> coxae (Attems 1928)	
<i>sd</i> seminal duct (Attems 1928)	
<i>bl</i> bladder wall (Attems 1928)	
<i>tp</i> tibial process (= <i>b</i> , Jeekel 1956)	
<i>tr</i> tracheal stalks (Attems 1928)	
<i>a</i> inflated basal lobe (Attems 1928)	
<i>dl</i> distal lamellae/process of telopodite (= <i>c</i> , <i>d</i> Jeekel 1956)	
<i>e</i> chitinous ridge (Attems 1928)	

IV. Reliable collection sites and dates when *Centrobolus* millipedes were sampled

Species	Locality	Date/s
<i>C. inscriptus</i>	Twinstreams, Mtunzini	¹ February 1996 December 1996
<i>C. annulatus</i>	Twinstreams, Mtunzini	February 1996 December 1996
<i>C. fulgidus</i>	Richards Bay Minerals St Lucia Estuary	February 1996 December 1996
<i>C. ruber</i>	Pt Shepstone	February 1996
<i>C. digrammus</i>	Simonstown waterfall	March 1997