

SEX IN SOUTHERN AFRICAN SPIROSTREPTIDA
MILLIPEDES: MECHANISMS OF SPERM COMPETITION
AND CRYPTIC FEMALE CHOICE

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A THESIS PRESENTED FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN THE ZOOLOGY DEPARTMENT
UNIVERSITY OF CAPE TOWN

FEBRUARY 1997

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ACKNOWLEDGMENTS

I am grateful to a large number of people who inspired, guided, assisted and supported me during the various phases of this work.

Sincere thanks to Mark Cooper, Mark Dangerfield, Willem Ferguson, Chido Mpamhanga and the many others who helped collect millipedes from around southern Africa, to Peter Croeser for help in animal identification and for the introduction to the South African millipede collection, and to Dr Leo Braak of the Kruger National Park for kind permission to work and collect animals in the park.

Thanks also to Solly, Lindy, Jess and Kira Levy for accommodation during the Zimbabwe fieldwork, to Prof. John Loveridge for the use of his laboratory space at the University of Zimbabwe, and to Prof. Gerhard van der Horst for the use of his laboratory space and photographic equipment at the University of the Western Cape.

To the staff of the Electron Microscopy Units at the Universities of Pretoria (Prof. Jan Coetzee and Chris van der Merwe) and Cape Town (Miranda Waldron, Dane Gerneke and especially Charlie Bruintjies), I am grateful for guidance and assistance in specimen preparation. Thanks also to Dane for initially teaching me to drive the S200.

Thanks to Babsie Potgieter and Wendy Sutton for advice and assistance in histological preparation, Adrian Baron for inspiration in micro-dissection techniques, to Brian Tibbles for initially introducing me to the use of radioisotopes and to Bruce Dell for technical assistance and the custom made light box. Special thanks to Neville Eden for all the technical support, photography and general patience with my inability to focus!

Thanks also to Mark Cooper for the millipede chats, Daniel Polakow for statistical advice, Veronique Suiro for translating the French literature, and especially to Andreas Tadler for sending me copies of much needed scarce papers, deciphering the German literature and helpful discussion.

In particular, I would like to thank my principal supervisor Dr Steven Telford for the many hours of discussion, assistance and guidance, and for his invaluable contribution to my academic development. Thanks also to my co-supervisors Dr Sue Nicolson and Prof. Alec Brown for administrative advice and support.

Finally, special thanks to my family and Dana, Dena, Franz and especially Marco for logistical, moral and spiritual support.

Financial support from the Universities of Cape Town and Pretoria and the F.R.D. is gratefully acknowledged.

ABSTRACT

Spirostreptida millipedes comprise three families, the Harpagophoridae, Spirostreptidae and Odontopygidae. They are polygynandrous. Males transfer sperm via species-specific accessory genitalia called gonopods, that comprise three components, two of which, the coxite and telopodite, are involved in processes of sperm transfer. The coxites function to translocate ejaculates from the penes to the vulvae, where they are stored. A delay between insemination and fertilisation provides an arena for syn- and postcopulatory sexual competition. These operate at the gametic level via sperm competition and cryptic female choice. By combining studies of genital form and function with single and double mating experiments, this study elucidates these processes in some southern African Spirostreptida millipedes.

Scanning electron and light microscopy are used to describe gonopod morphology for 26 Spirostreptida species (6 Harpagophoridae, 13 Spirostreptidae, 7 Odontopygidae). For five of these, gonopod functional morphology is also described. The association of gonopod components is similar within families and more particularly within genera, and it is predicted that the functional morphology and mechanisms of competition are conserved within these groups. With the exception of the Spirostreptidae coxites, which are spined, Spirostreptida telopodites are the most complex regions of the gonopods. Gonopod form and function is not accounted for by sperm transfer alone. Structural evidence implicates both cryptic female choice and sperm competition in their evolution. (Chapter 2).

Sperm morphology is described for 18 species (8 Harpagophoridae, 7 Spirostreptidae, 3 Odontopygidae) using bright field and phase contrast microscopy. Sperm are non-motile and either disc- or triangle-shaped. Sperm immotility has implications for the mechanisms of competition because it precludes independent sperm movement into or within the sperm stores (Chapter 2).

External vulval morphology is described for 20 Spirostreptida species (8 Spirostreptidae spp; 6 Harpagophoridae spp; 6 Odontopygidae spp), and a detailed histological account is provided for representative taxa of each family. In all three families, bursae are located in deeply invaginated vulval sacs. Sperm are stored in the bursae in a series of interconnecting ampullae that are associated with bursal glands and

muscles. Muscles fan out from the spermathecae to the bursal walls. Bursal muscles may "sanction" cryptic female choice via control of ejaculate storage and manipulation. In *A. uncinatus* (Spirostreptidae), females store sperm for protracted periods and the non-gametic component of the ejaculate, the granules, may function as mating plugs.

In the Harpagophoridae, bursae protrude from the gonopore. However, the spermathecal ampullae themselves are not directly accessible to the gonopods because the distal telopodites are broader than they are. In both the Spirostreptidae and Odontopygidae, bursae are situated at the bottom of the vulval sacs, some distance from the gonopores. In spite of this, Spirostreptidae telopodites reach the bursal furrow that gives rise to the spermathecal ampullae. Due to the orientation of the bursae and the size of the distal telopodites, gonopods do not enter the ampullae. The orientation of the bursae and their distance from the gonopore suggest that Odontopygidae telopodites do not have direct access to the sperm stores either (Chapter 3).

Processes of ejaculate transfer are quantified for two Spirostreptida species, *Alloporus uncinatus* (Spirostreptidae) and *Poratophilus diplodontus* (Harpagophoridae). By radiolabelling ejaculates with tritiated thymidine, and separating copula pairs at varying time intervals from the onset of copulation, it is shown that sperm transfer occurs at the beginning of copulation and the proportion of ejaculate at the bottom of the vulvae increases with time. Early insemination has implications for the mechanisms of competition because males cannot manipulate rival ejaculates without also affecting their own. The adaptive significance of prolonging copulation beyond insemination is discussed (Chapter 4).

Radioactive labelling techniques are used to examine mechanisms of competition, and to test whether sperm storage is affected by a temporal delay between successive matings (*P. diplodontus* and *A. uncinatus*). Because P_2 cannot be inferred from a measure of ejaculate volume, the term V_2 is proposed to describe the proportion of ejaculate contributed to the sperm stores of the female by the second of two males to mate with her. Genital manipulation experiments were performed to test the hypothesis that telopodites function in ejaculate placement and displacement.

In *P. diplodontus*, $V_2 \approx 0.62$ following a double mating. Ejaculate storage is not affected by a 24 hour delay between matings. Vulval capacity is reached with single ejaculates and for subsequent ones to be accommodated, at least 64.52% of prior

ejaculates must be removed. Removal is partially effected by the distal telopodites (26.46%) but is not totally accounted for by direct male processes. The balance may be effected by ejaculate flushing, a strategy that concurs with smaller ejaculate volumes remaining within the vulvae than are initially transferred. Partial removal may be a consequence of both the early onset of insemination (males would be unable to remove rival ejaculates without also affecting their own) and the storage of ejaculates in inaccessible spermathecal ampullae (Chapter 5).

In *A. uncinatus*, vulval capacity is greater than that of *P. diplodontus* and single ejaculates are apparently too small to fill the vulvae. Coincident with this is a greater number of spermathecal ampullae. Later males do not affect prior ejaculates. Following a double mating $V_2 \approx 0.5$, unless a temporal delay is imposed between matings in which case females have been shown to reduce the contribution of the first male and $V_2 \rightarrow 0.71$. Spirostreptidae telopodites are implicated in ejaculate transfer, but not in the movement of ejaculates to the bottom of the vulvae. Only in the Spirostreptidae does complete telopodite retraction bring the distal telopodite in contact with the coxite, and the coxal spines may function to temporarily suspend the sperm during copulation (Chapter 6).

Preliminary data from mating experiments on the Odontopygidae are discussed. Patterns of insemination are similar to those of *A. uncinatus* and *P. diplodontus*. In double mating experiments using radiolabelling in *Chaleponcus digitatus*, $V_2 \approx 0.5$. The same was true of *Spinotarsus* sp.1. A result of first male precedence in this species may have been obscured by small sample sizes (Appendix D).

Several male- and female-mediated processes produce the observed patterns of ejaculate transfer and storage in the Spirostreptida. The evolution of inter-family differences in Spirostreptida genital morphology and the usefulness of vulval morphology as predictors of male- and female-mediated processes of competition are discussed. This work highlights the notion that insight into the mechanisms of competition is best achieved by combining studies of both male and female genital functional morphology.

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CHAPTER ONE

INTRODUCTION

1.1 Sexual selection

Sexual selection was first proposed to rationalise the evolution of elaborate male characters that seemed contradictory to survival (Darwin 1871). Driven by variation in individual reproductive success, it operates through two distinct processes: (1) competition within a sex for access to members of the opposite sex (intrasexual selection or intrasexual competition), and (2) preferences of individuals of one sex for particular members of the opposite sex (intersexual selection or epigamic choice). These processes are related to intersexual differences in parental investment, both in terms of anisogamy and parental care (Bateman 1948; Fisher 1958; Trivers 1972). Individuals of the sex investing less (usually males) generally compete for access to mates, while individuals of the sex investing more (usually females) are expected to exercise more stringent mate choice.

Historically studies of sexual selection have focused on precopulatory mechanisms of sexual competition (e.g. Andersson 1994). Because copulation does not automatically lead to fertilisation (Eberhard 1985), sexual selection must also involve processes of syn- and postcopulatory competition. These processes operate at the gametic level via sperm competition (Parker 1970a) and "cryptic" female choice (Thornhill 1983).

1.2 Sperm competition

Sperm competition was first defined as the "competition within a single female between the sperm of two or more males for the fertilisation of the ova" (Parker 1970a). Where females are multiply mated during a single reproductive cycle and sperm are stored, the

sperm of different males may overlap spatially and temporally within the female reproductive tract, and compete directly for the fertilisation of ova. It is a powerful selective force in the evolution of reproductive behaviour and genital morphology (Parker 1970a; Smith 1984; Birkhead 1989) generating two sets of opposing adaptations; (a) those that enhance an individual's ability to pre-empt previous ejaculates and (b) those that facilitate or "resist" ejaculate pre-emption (Parker 1970a; Smith 1984).

a. Adaptations related to the pre-emption of previous ejaculates include ejaculate dilution (Parker 1970a). If the sperm of different males have equal fertilisation potential (this is not always the case, see Martin *et al.* 1974; Sivinski 1984) and mix completely prior to oviposition such that they are utilised for fertilisation in proportion to their numbers, a raffle ensues (Birkhead and Møller 1992). Simply inseminating a female results in the dilution of previous ejaculates; and larger ejaculates confer a numerical superiority and consequently a fertilisation advantage (e.g. the cockroach *Diploptera punctata*, Woodhead 1985; the field cricket *Gryllus bimaculatus*, Simmons 1986, 1987a; the milkweed leaf beetle *Labidomera clivicollis clivicollis*, Dickinson 1986; the beetle *Callosobruchus maculatus*, Eady 1995).

However, contrary to Trivers (1972), the cost of producing sperm is not negligible (Dewsbury 1982) and males do not simply increase ejaculate size to optimise paternity. As predicted by Parker (1970a; 1990a, b), males rationalise sperm utilisation by manipulating ejaculate size according to the probability that their sperm find themselves in competition with rival sperm (Baker and Bellis 1989; Gage and Baker 1991; Gage 1991). Males also improve their reproductive success while lowering the costs of overall sperm production by displacing rival sperm and improving the chances that their own sperm meet up with emerging ova. This is achieved in two ways (Birkhead and Hunter 1990): (1) through the repositioning of rival ejaculates within the stores such that they do not have access to emerging ova, and (2) through the removal of rival ejaculates from the stores.

1. Repositioning can occur through the displacement of rival ejaculates from the primary storage sites: In the silkworm *Bombyx mori* the sperm mass of the second male pushes that of the first male away from the entrance to the spermathecae (Suzuki *et al.* 1996), and in the mite *Caloglyphus berlesei*, prior to sperm transfer, rival sperm are pushed away from the efferent ducts leading from the spermatheca to ovaries (Radwan

and Witalinski 1991). The fact that the spermathecae are inaccessible to the male genitalia suggests that they cannot be directly responsible for redistribution and a female role has therefore been proposed (see Eberhard 1996 p39). Prior ejaculates can also be "stratified" behind later ones. Stratification is partially dependent on the morphology of the female's sperm storage organs (Schlager 1960; Walker 1980): where the oviduct inserts above the entrance of the spermathecae, as in insects, sperm manipulation can be achieved via distal packing (e.g. the waterbug *Abedus herberti*, Smith 1979; the dragonfly *Crocothemis erythraea*, Siva-Jothy 1988; the dragonfly *Nanophya pygmaea*, Siva-Jothy and Tsubaki 1994). Where insertion is below, such that emerging ova pass through "conduit" sperm stores (Austad 1984), stratification is structurally precluded because ova first make contact with sperm placed close to the bottom of the stores (e.g. the Sierra dome spider *Linyphia litigiosa*, Watson 1991; Acari, Thomas and Zeh 1984; Austad 1984).

2. In some taxa, ejaculate removal is effected directly by the male genitalia. Waage (1979a) was the first to attribute a sperm removal function to an intromittent organ of an animal. Working on the damselfly *Calopteryx maculata*, he demonstrated that during copulation, but prior to insemination, spines on the penis entrap sperm of rival males and remove them from the sperm storage organs of the female. Recurved spines, bristles and barbs on male genitalia have since been shown to scoop or brush rival sperm from the female's sperm storage organs in several other Odonata (Miller and Miller 1981; Fincke 1984; McVey and Smittle 1984; Waage 1984, 1986a, b; Miller 1987a, b, 1990; Siva-Jothy 1987a; Michiels and Dhont 1988; Siva-Jothy and Tsubaki 1989a; Cordero and Miller 1992). Two beetles, *Tenebrio molitor* and *Psacotheta hilaris*, are the only non-Odonata in which direct removal has been demonstrated (Yokoi 1990 cited in Eberhard 1996; Gage 1992).

Where males do not have direct access to the sperm stores, removal can be effected indirectly via volumetric displacement or ejaculate flushing (Thornhill and Alcock 1983; Parker 1984; *Scatophaga stercoraria* dung flies, Parker 1970b; the hemipteran *Onocopeltus fasciatus*, Economopoulos and Gordon 1972; *Drosophila melanogaster*, Scott and Williams 1992). For example, in the tree cricket *Trujalia hibinonis* prior sperm are believed to be flushed from the stores by the ejaculate of the last male (Ono *et al.* 1989). Indirect removal via the induction of females to discard rival sperm has also been documented (Eberhard 1996). In the carrion fly *Dryomyza anilis*, immediately after copulating with a female the male performs a series of tapping

sequences on the female's external genitalia, causing her to extrude a droplet of (mostly) rival sperm (Otronen and Siva-Jothy 1991).

The pre-emption of previous ejaculates via destruction and inactivation has also been proposed (Gromko *et al.* 1984; "killer sperm", Silberglied *et al.* 1984; "kamakazi sperm", Baker and Bellis 1988).

b. Adaptations to avoid potential loss of progeny sustained by future inseminations include blocking female genital openings with copulatory plugs (Parker 1970a; Aiken 1992), mate guarding (Parker 1974; Waage 1979b; Thornhill and Alcock 1983) and the induction of non-receptivity (Eberhard 1996).

Prolonged copulation can also function as a sperm competition avoidance mechanism (Parker 1970b; Thornhill and Alcock 1983, e.g. the hemipteran *Nezara viridula*, McLain 1980; the heteropteran *Lygaea equestris*, Sillen-Tullberg 1981; milkweed beetles *Labidomera clivicollis*, Dickinson 1986, the millipede *Alloporus uncinatus* Telford and Dangerfield 1990a). In this context, increases in the duration of copulation in response to increasing male bias in the operational sex ratio, have been interpreted both as a form of mate guarding (Telford and Dangerfield 1990a Clark 1988), and in the induction of refractory behaviour (Eberhard 1996), both of which would be more crucial with other males already present. The induction of non-receptivity can also be achieved through the transfer of large ejaculates (e.g. monarch butterflies *Danaus plexippus*, Oberhauser 1989; *Callosobruchus maculatus* beetles, Eady 1995).

1.3 Cryptic female choice

Although male behaviours associated with sperm competition are more obvious than those of females, and are therefore more readily studied (Birkhead and Møller 1992), the evolutionary perspective of females is critical to understanding mechanisms of competition. In most taxa, sperm competition takes place within the female reproductive tract, as do processes of sperm transport, storage and fertilisation. Anatomical and physiological control over the arena of competition may afford females the ultimate control of paternity via "cryptic" choice (Thornhill 1983; Eberhard 1996).

One of the suggested benefits of multiple mating¹ to females is the promotion of direct sperm competition, because effective pre-emption will be inherited by their sons (Knowlton and Greenwell 1984; Birkhead and Møller 1992). Alternative explanations may be found in cryptic choice: for example, where the cost of multiple mating is less than the cost of rejection, syn- and postcopulatory cues may be the basis on which females screen males (Sivinski 1984; Eberhard 1996).

Syn- and postcopulatory mechanisms of female choice are well documented (Sivinski 1980; 1984; Walker 1980; Thornhill 1984; see Eberhard 1996 for an extensive review):

a. Where the number of sperm transferred covaries with copulation duration, females can control sperm transfer (Eberhard 1996) by prematurely terminating copulation (Austad 1984; Heming-van Battum and Heming 1986; Birkhead and Møller 1992) or spermatophore attachment time (e.g. the cricket *Gryllus bimaculatus*, Simmons 1987b).

b. Females can select between ejaculates. In most taxa males do not have direct access to the sperm stores (Eberhard 1985) and sperm are moved from insemination to storage, and from storage to fertilisation sites (Eberhard 1996). These are often female-mediated processes (Eberhard 1996), effected by active transport systems (e.g. sophisticated musculature in the boll weevil *Anthonomus grandis*, Villavaso 1974, 1975a; fluid withdrawal from the spermathecae in the biting midge *Culicoides melleus*, Linley 1981, Linley and Simmons 1981; drawing of sperm into spermathecae using dilator muscles in *Herbrus pusillus* and *H. ruficeps* bugs, Heming-van Battum and Heming 1986).

It has been suggested that females use naturally selected transport systems to selectively utilise, store and expel ejaculates of different males (Eberhard 1996; e.g. selective utilisation: the Sierra dome spider *Linyphia litigiosa*, Watson 1991; selective storage: the dung fly *Scathophaga stercoraria*, Ward 1993; the beetle *Chelymorpha alternans*, Rodriguez 1994; selective expulsion: *Spodoptera litura*, Etman and Hooper 1979; the fly *Dryomyza anilis*, Otronen 1990; Otronen and Siva-Jothy 1991; the bushcricket *Metaplastes ornatus*, von Helversen and von Helversen 1991). Also documented are ejaculate absorption (e.g. in the nannoptera species group, Pitnick *et*

¹ see Walker 1980; Thornhill and Alcock 1983; Halliday and Arnold 1987; Arnqvist 1989; Hunter *et al.* 1993 for detailed discussion of the costs and benefits of multiple mating.

al. 1991) and destruction (Eberhard 1985; e.g. the melon fly *Dacus cucurbitae*, Yamagishi *et al.* 1992).

Copulatory courtship is believed to comprise syn- and postcopulatory behavioural, physiological and morphological cues (Eberhard 1994; 1996). Genital functional morphology is frequently cited as stimulatory (Walker 1980; Eberhard 1985; 1996; see Eberhard 1994 for an overview). Examples include the pumping movements that accompany sperm transfer in the Odonata (e.g. the dragonfly *Ischnura verticalis*, Eberhard 1996 citing Fincke 1987), and the rhythmic movements that precede sperm transfer in the millipede *Cylindroiulus punctuatus* (Eberhard 1996 citing Haaker and Fuchs 1970). In this context, runaway selection has been invoked to explain genital complexity (Eberhard 1985; Fisher 1958).

In arthropods, spermathecae range from relatively simple structures to complex convoluted organs that restrict access of male genitalia to sites of sperm storage (Snodgrass 1935; Matsuda 1976; Eberhard 1985; Kaulenas 1992). Multiple sperm stores may facilitate the creation of primary and secondary storage sites (Eberhard 1996): in the dung fly *Scathophaga stercoraria*, females have three spermathecae in which sperm are unevenly distributed, and cryptic preferences for larger males have been related to sperm distribution patterns (Ward 1993). In some taxa, males have direct access to parts of the storage areas while others remain inaccessible (e.g. the carrion fly *Dryomyza anilis*, Otronen and Siva-Jothy 1991; the yellow dung fly *Scathophaga stercoraria*, Ward 1993; the Namib beetle *Onymacris unguicularis*, de Villiers and Hanrahan 1991; Libellulidae, Miller 1991), facilitating both the promotion of direct competition (Birkhead and Møller 1992) and the creation of a sperm cache (*sensu* Siva-Jothy and Hooper 1995). In the damselfly *Calopteryx splendens xanthostoma*, sperm diversity in the spermathecae is greater than that of the bursa copulatrix, and males have direct access to the bursa, but not to the spermathecae (Siva-Jothy and Hooper 1995).

1.4 Sperm precedence

Sperm precedence is a measure of the reproductive output of males. It is the product of both sperm competition and cryptic female choice on processes of insemination and fertilisation. Many studies present mean values for P_2 (the proportion of offspring sired by the last male to mate in a double mating sequence), and show that although first

male and incomplete sperm precedence occur, the last male to mate frequently fertilises most of the offspring (Parker 1970b; Gwynne 1984; Ridley 1989). However, and importantly, P_2 values (a) show considerable intraspecific variation (Fincke 1984; Lewis and Austad 1990; Simmons and Parker 1992; Eady 1994a; Conner 1995), (b) shift with varying temporal delays between matings and between the end of copulation and oviposition (Retnakaran 1974; Boorman and Parker 1976; Gromko *et al.* 1984; Siva-Jothy and Tsubaki 1989, 1994; Otronen 1990; Tsubaki and Yamagishi 1991; Yamagishi *et al.* 1992), and (c) are affected by mate number (Zeh and Zeh 1994).

Intraspecific variation in P_2 values has been attributed to variance between competing males in terms of sperm number transferred and displaced, body size, copulation duration and behaviour (e.g. variation in sperm number transferred: Dickinson 1986; Baker and Bellis 1989; Bellis *et al.* 1990; Cordero and Miller 1992; sperm number displaced: Parker 1970b; Siva-Jothy 1987a; body size: Berrigan and Locke 1991; Simmons and Parker 1992; copulation duration: Vollrath 1980 cited Austad 1984; Rubenstein 1989; Wolf *et al.* 1989; Lewis and Austad 1990; Simmons and Parker 1992; behaviour: Otronen and Siva-Jothy 1991). Variation in sperm precedence has also been related to sperm mortality (Tsubaki and Yamagishi 1991; Olsson *et al.* 1994), and differential fertilisation abilities of the sperm (Martin *et al.* 1974; Dewsbury 1984; Sivinski 1984).

The other sources of P_2 variation (temporal variation in last male precedence and changes related to mate number) are associated with changes in sperm distribution patterns within the sperm stores (Retnakaran 1974; Siva-Jothy and Tsubaki 1989a, b; Gage 1992; Zeh and Zeh 1994; Suzuki *et al.* 1996). Two mechanisms have been suggested:

a. Changes in P_2 can ensue from initial stratification and subsequent mixing of ejaculates (Schlager 1960; the dragonfly *Erythemis simplicicollis*, McVey and Smittle 1984; the damselfly *Mnais pruinosa pruinosa*, Siva-Jothy and Tsubaki 1989; the harlequin beetle-riding pseudoscorpion *Cordylochernes scorpioides*, Zeh and Zeh 1994). Mixing may be effected by the ejaculates of later males or may occur with time. For example, in *Cordylochernes scorpioides*, double matings result in last male sperm precedence. However, if a third mating follows, all males have equal success of paternity. These results were explained by making certain assumptions about how ejaculates redistribute with each new insemination. It is argued that in double mating

experiments, last male precedence is a consequence of stratification within the spermathecae, but when spermathecae are filled to capacity, stratification breaks down because sufficient pressure is generated to cause sperm mixing (Zeh and Zeh 1994). In the damselfly *Mnais pruinosa pruinosa*, last males have almost complete precedence immediately following mating, irrespective of the amount of rival sperm displaced. However, when rival sperm are incompletely removed, a delay between insemination and oviposition results in sperm mixing (Siva-Jothy and Tsubaki 1989)

b. Changes in P_2 can also result from sperm moving from accessible regions of the stores to those where the second male cannot reach them (Retnakaran 1974; Radwan 1991; Gage 1992; Ward 1993). In the spruce budworm *Choristoneura fumiferana* the male transfers a spermatophore to the bursa of the female during copulation, and sperm move from the bursa to the seminal receptacle. If a female mates with a second male before the sperm of the first male enter the seminal receptacle, sperm mixing occurs. If complete translocation is already complete, first male precedence operates (Retnakaran 1974). A similar pattern has been shown in the beetle *Tenebrio molitor*, where the number of sperm removed by the last male declines with an increase in the inter-mating interval. This decline was attributed to the migration of sperm from the bursa to the spermatheca, where they are not accessible to the male's genitalia (Gage 1992).

Female preferences in sperm manipulation and utilisation (Eberhard 1985; 1996; Rubenstein 1989; Eady 1994a; Conner 1995; see above) can impact on ejaculate distribution patterns and also potentially affect P_2 .

1.5 Mechanisms of competition

Recently the emphasis has shifted from pattern to process, i.e. from describing patterns of precedence to identifying the mechanisms conferring them, allowing for the formulation of *a priori* predictions of precedence patterns and the assessment of the roles of males and females.

Several mathematical models that are based on P_2 values, sperm transfer and anatomy have been formulated to test predictions about mechanisms of competition (e.g. Lessells and Birkhead 1990; Parker *et al.* 1990; Parker and Simmons 1991). However, because the mechanisms are generally poorly understood, the models are frequently based on assumptions (e.g. unit for unit displacement from the stores, Parker *et al.*

1990, later modified in Parker and Simmons 1991) and often fail to incorporate female-mediated processes (Ward 1993; Eady 1995). Accurate predictions about mechanisms are dependent on cognisance of patterns of sperm transfer and subsequent distribution, and genital anatomy and function.

Several studies have determined the mechanisms of competition by manipulating copulatory behaviour, and monitoring subsequent ejaculate distribution patterns within the sperm stores and on the male genitalia (e.g. the damselflies *Calopteryx maculata*, Waage 1979a; *Enallagma hageni*, Fincke 1984; *Mnais pruinosa pruinosa*, Siva-Jothy and Tsubaki 1989a, b; Siva-Jothy 1984, 1987b; *Ischnura graellsii*, Cordero and Miller 1992; the flesh fly *Neobellieria bullata*, Berrigan and Locke 1991; the bush cricket *Metaplastes ornatus*, von Helversen and von Helversen 1991; the acarid mite *Caloglyphus berlesei*, Radwan and Witalinski 1991; the mealworm beetle *Tenebrio molitor*, Gage 1992; the beetle *Callosobruchus maculatus*, Eady 1994b). In some cases, these observations have allowed workers to elucidate the functional morphology of the genitalia (e.g. Waage 1979a; Gage 1992).

Genital manipulations have also been used to empirically demonstrate the role of the genitalia: By severing the spermathecal muscles in the boll weevil *Anthonomus grandis* it was shown that sperm displacement was female-mediated (Villavaso 1975). By blocking the ejaculatory ducts of the tree cricket *Truljalia hibinonis* rival sperm are flushed from the female's sperm stores by the ejaculate of the last male (Ono *et al.* 1989).

To distinguish between ejaculates of different males within the sperm stores requires the implementation of direct labelling techniques. These have included radiolabelling (Retnakaran 1971; 1974; Villavaso 1975a; Otronen and Siva-Jothy 1991) and the use of fluorescent (Birkhead and Møller 1992) and vital dyes (*Truljalia hibinonis*, Ono *et al.* 1989; *Bombyx mori*, Suzuki *et al.* 1996). Although the interpretation of patterns based on these techniques is still rudimentary (Birkhead and Møller 1992), these techniques have been used to measure volumetric contributions of males (Villavaso 1975a) and ejaculate distribution patterns (e.g. the boll weevil *Anthonomus grandis*, Villavaso 1975a; domestic fowl, van Krey *et al.* 1980; the acarid mite *Caloglyphus berlesei*, Radwan and Witalinski 1991). In the carrion fly *Dryomyza anilis*, females mate with several males and the last male to mate can achieve a fertilisation precedence of 70%. Immediately after copulating with a female the male performs a series of

tapping sequences on the female's external genitalia using his genital claspers whilst simultaneously pressing her abdomen with his hind legs. The female responds to this behaviour by extruding a droplet of sperm. By labelling ejaculates with radioisotopes, it was demonstrated that the droplet comprised sperm mostly from the previous male (Otronen and Siva-Jothy 1991).

1.6 Millipedes

The estimated 70 000 extant species of millipede comprise a major element of the soil fauna in both tropical and temperate ecosystems (Blower 1985). Spirostreptida millipedes are well represented throughout the Southern African subregion and comprise three families, the Harpagophoridae, Odontopygidae and Spirostreptidae (see Appendix A for Diplopoda classification). They are sexually dimorphic, with males smaller and more slender than females, although the degree of dimorphism varies (see later).

Spirostreptida millipedes are seasonally active and during the rainy months are particularly conspicuous in the dry, savannah habitats of Southern Africa (Lawrence 1984). Surface activity is related to feeding and reproduction (Dangerfield and Telford 1991; Dangerfield *et al.* 1993) and during these periods, population densities can be extremely high (Dangerfield and Telford 1989; Telford and Dangerfield 1994).

Females usually emerge first and as the breeding season progresses, the operational sex ratio (OSR) (Emlin and Oring 1977) becomes increasingly male-biased (Dangerfield and Telford 1990a). Millipedes are polygynandrous (Telford and Dangerfield 1990a, b) and store sperm for long periods (Snider 1981; Hopkin and Read 1992). These prerequisites for sperm competition combined with high population densities and male biased OSR's, propound intense mate competition.

Studies of diplopod mating behaviour have shown a diversity of patterns associated with courtship, mating and sperm transfer (Haaker 1974; Hopkin and Read 1992; Telford and Dangerfield 1993a, b). Males initiate copulation by approaching females from behind and moving towards the head along the dorsal surface (Mauriès 1969, pers. obs). In most Spirostreptida species, the male coils his body around the female (Plate 1.1). *Calostreptus carinatus* and *Bicoxidens matabele* copulate in parallel (Telford and Dangerfield 1993b, pers. obs). Mate recognition is believed to be the



PLATE 1.1 Copula pair of *Alloporus uncinatus* (Spirostreptidae) millipedes. The male is coiled around the female. A translucent sac (indicated by arrow), that is filled with haemolymph, expands at the base of the gonopods during eversion. It remains expanded for the duration of copulation. m = male.

consequence of a random walk (Telford and Dangerfield 1993b), and based on a combination of tactile (Mukhopadhyaya and Bhakat 1983) and chemical cues (Haaker 1969; Carey and Bull 1986). In the Spirostreptidae the onset of copulation is associated with females attaching their mouthparts to species-specific prefemoral projections at the base of the male's first pair of legs (Attems 1950; Schubart 1966; Enghoff 1990; e.g. *Archispirostreptus gigas* (Spirostreptidae), Krabbe 1979).

Females initiate avoidance behaviour shortly after males make contact with them, but cannot always prevent copulation (Haaker and Fuchs 1970; Mukhopadhyaya and Saha 1981; Tadler 1996; pers. obs.). In the Spirostreptidae species *Alloporus uncinatus*, a large male snapped the head off a female in an attempt to copulate with her (Telford and Dangerfield 1993a).

Gonopores are paired structures that occur on the third body segment, just behind the second pair of legs (Blower 1985, Fig. 1.1). In females, they open into paired vulvae that are attached to the base of the second leg pair (Blower 1985; Hopkin and Read 1992). In males they terminate in penes from which sperm are released. In addition to gonopores, males bear a pair of structurally complex intromittent genitalia called

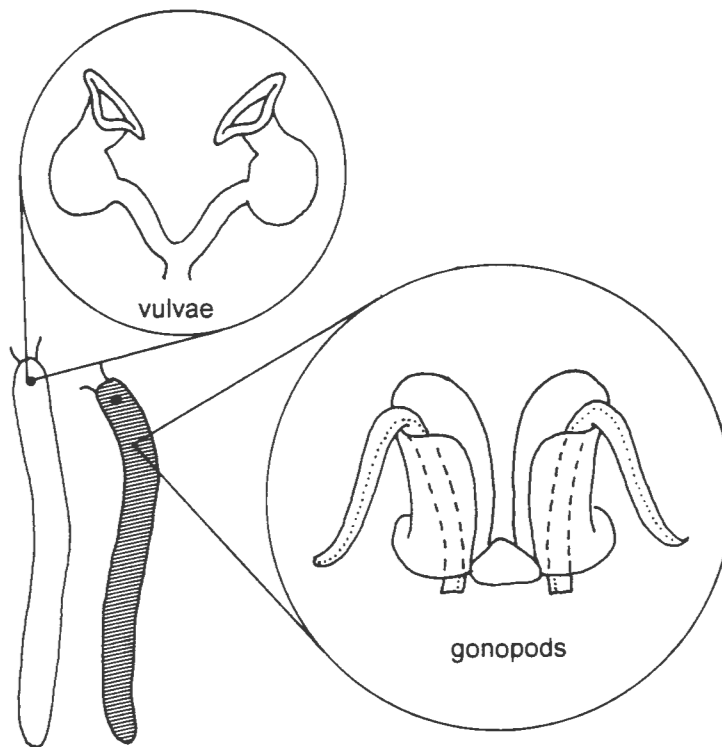


FIGURE 1.1 Diagrammatic representation of male and female millipedes and the position of gonopods and vulvae.

□ = female; ▨ = male; • = gonopores (second segment).

gonopods on their seventh body segment. These structures are normally drawn into the body so that only their distal ends are visible.

During copulation, the gonopods are everted and a translucent sac, that is filled with haemolymph, expands at their base (Plate 1.1). The significance of this sac has not been explained. However, as gonopod eversion is always accompanied by inflation of this sac, it is speculated that the sac may play a role in their eversion. It may also play a role in increasing male genital size - inflatable genitalia have been suggested to be adaptive in that they increase genital size without affecting their ability to be accommodated within the body of the male.

Sperm are translocated via the gonopods from the penes to the vulvae where they are stored (Krabbe 1982; Blower 1985; Barnes 1986, Hopkin and Read 1992). Gonopods have also previously been suggested to function in vulval cleaning (Haaker and Fuchs 1970), sperm removal (Barnett 1991; Barnett *et al.* 1993; Tadler 1996) and cryptic female choice (Eberhard 1996; Tadler 1996). Copulation duration is usually prolonged,

and differs between species and between populations of the same species (Berkowitz and Warburg 1988; Telford and Dangerfield 1990a; this work).

Following copulation, pairs separate and both males and females groom their genitalia. Fertilisation is coupled to oviposition and the process is elaborate (Bhakat *et al.* 1989; Hopkin and Read 1992).

1.7 Scope of this thesis

The aim of this study is to elucidate the mechanisms of competition in millipedes of the order Spirostreptida, by combining studies of genital morphology with mating experiments using radiolabelled ejaculates. Paternity studies were not conducted due to difficulty in inducing oviposition in the laboratory.

Genital form and function of the Harpagophoridae, Spirostreptidae and Odontopygidae are examined in Chapters 2 and 3. These descriptive studies are an essential first step towards understanding the function of these complex structures, and allow *a priori* predictions to be made about mechanisms of sperm displacement and patterns of sperm precedence. They form the basis for beginning an iterative series of experiments and manipulations designed to quantify mechanisms of sperm competition.

In Chapter 4 the timing of insemination in *Poratophilus dipodontus* (Harpagophoridae) and *Alloporus uncinatus* (Spirostreptidae) is established by interrupting copulations and using tritiated thymidine to label ejaculates. The implications of the timing of insemination on the mechanisms of competition are discussed.

Patterns of ejaculate transfer and subsequent distribution in *P. dipodontus* and *A. uncinatus* are examined in Chapters 5 and 6 respectively. Ejaculate radiolabelling is combined with double mating experiments to elucidate processes of sperm storage, and genital manipulations are performed to empirically test genital function. A new term, V_2 , is proposed to describe the relationship between male ejaculates within the sperm stores.

In Chapter 7 the key results are summarised, the mechanisms of ejaculate competition in the Spirostreptida are discussed, and evolutionary perspectives are considered.

1.8 Animal collection, maintenance and identification

Animals were collected from various sites in southern Africa (see Appendix B), and housed in unisex groups in the laboratory (25°C; 75% relative humidity) in vermiculite-lined glass aquaria (50 x 30 x 50 cm). Food in the form of fresh vegetables was provided *ad libitum*. Species were identified according to Spirostreptida taxonomic literature (Attems 1914, 1928, 1934, 1937; Schubart 1966; Krabbe 1982; Kraus 1960, 1966) using body characters, gonopod morphology and distribution patterns. Where identification to species level was not possible, the genus is provided.

CHAPTER TWO

GONOPOD FUNCTIONAL MORPHOLOGY AND SPERM MORPHOLOGY: IMPLICATIONS FOR THE MECHANISMS OF COMPETITION

2.1 Introduction

Selection for efficient transfer of sperm does not account for the evolution of the elaborate male genitalic appendages that are a widespread trait in many animals with internal fertilization (Eberhard 1985). Historically, genital complexity has been interpreted as a consequence of several processes including tactile and mechanical processes of mate recognition and pleiotropy (Kraus 1968; see Eberhard 1985 for a detailed account). More recently, it has been explained in the context of sexual selection. Eberhard (1985) argued that genitalia are never used directly in male-male interactions and therefore, as opposed to other secondary sexual characteristics, genital evolution is driven exclusively by female choice. While Eberhard's generalisation about genital complexity being exclusively a process of female choice may hold for most sexual interactions, it is complicated if sexual selection via sperm competition occurs. Where females are multiply mated and store sperm, and fertilisation is delayed (the prerequisite criteria for sperm competition to occur, Parker 1970a), genitalia may have evolved as devices to actuate last-male fertilization precedence (Parker 1970a; Waage 1982; 1984; Thornhill and Alcock 1983; Gwynne 1984; Gage 1992).

2.1.1 Gonopod functional morphology

Gonopods are taxonomic characters of primary importance in many millipedes and accordingly their morphology is well documented (Attems 1914; 1928; Hopkin and Read 1992; Kraus 1960; 1966). However, only recently have the selective processes responsible for the evolution of these complex structures been considered (Tadler 1993; 1996, but see Kraus 1968 on pleiotropy; Verhoef 1926 - 1932 on the lock and

key hypothesis, cited in Tadler 1993, critique by Tadler 1993). Because sperm transfer is accomplished by millipedes bearing simple gonopods (e.g. the Colobognatha, Blower 1985; Hopkin and Read 1992) and those without gonopods (e.g. bristle millipedes (*Polyxenus* spp., Polyxenida, Blower 1985; Hopkin and Read 1992), sperm transfer alone cannot account for gonopod complexity. Recently it has been interpreted in the context of sexual competition (Barnett 1991; Barnett *et al.* 1993; Tadler 1993; Tadler 1996).

2.1.2 Previous work on the functional morphology of Diplopoda genitalia

Gonopod functional morphology differs significantly between the Diplopod orders, and in each order different components are involved in processes of sperm transfer. In most Diplopod orders, both pairs of legs on the seventh segment form the gonopods, and sperm transfer is accomplished by the anterior gonopods. For example, in the Chordeumatida (Fig. 2.1a, b), the posterior gonopods are fused with the coxite to form the podosternite, and function to 'stabilise' (sensu Tadler 1996) the bursa during sperm transfer (Tadler 1993). The anterior gonopods comprise two components, the cheirite¹ and syncoxite. The cheirite is moveable and functions to pull the bursae from the vulval sacs. The syncoxite functions in sperm transfer: sperm are transferred from vesicles at the base of the syncoxite via the lateral brushes to the bursae. During copulation the gonopods and bursae are juxtaposed, and this has been suggested to be significant in female choice (Tadler 1993).

The Julida differ from other Diplopoda in that sperm transfer is performed by the *posterior* gonopods (this is believed to be a key step in the evolution of the Julida, Read 1990). In the family Parajulidae, sperm are translocated to the vulvae by the posterior gonopods. The anterior gonopods function to translocate sperm from the penes to the posterior gonopods (Matthews and Bultman 1993). In the Julida family Nematsomatidae (Fig. 2.1c), the anterior gonopods also have no contact with the bursae. A flagellum arises at their base and passes into a groove on the posterior gonopods. Sperm are transferred from the distal ends of the posterior gonopods to the bursae. In Julid millipedes gonopods are inserted twice, once prior to sperm loading (Haaker and Fuchs 1970). The first insertion of the gonopods is associated with rhythmic movements of the flagellae that were initially believed to "clean" the bursae

¹ The clasping hook of the cheirite is considered to be homologous with the telopodite of the seventh pair of legs (Verhoef 1926-32 cited Tadler 1993).

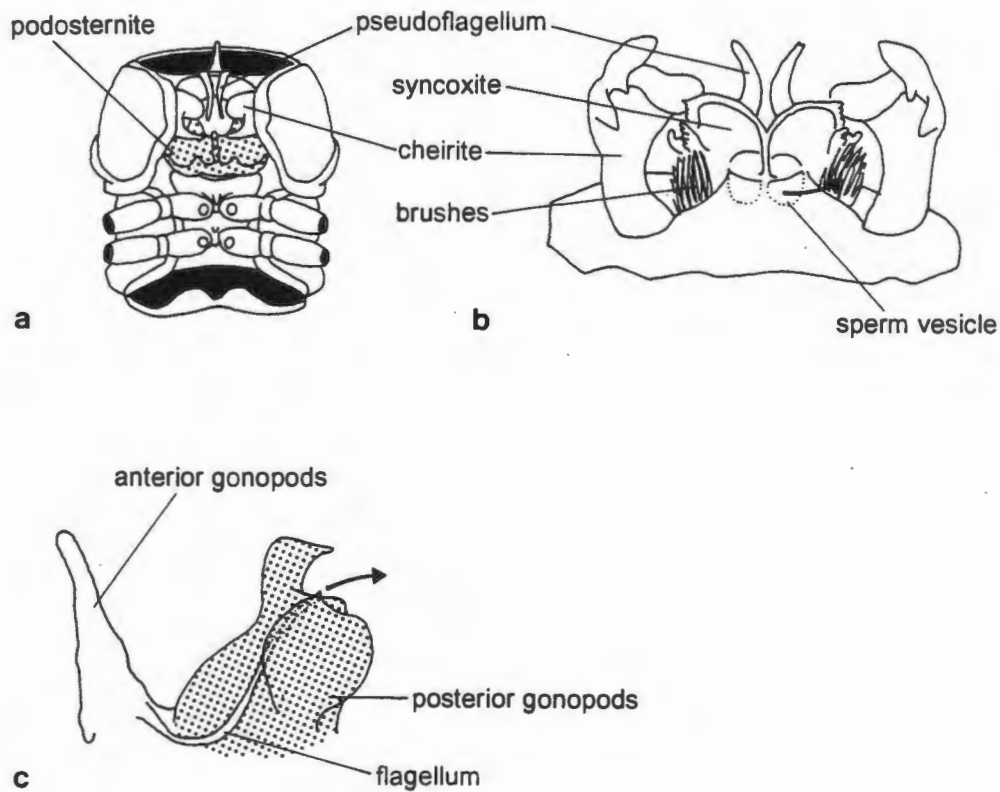


FIGURE 2.1 Diagrammatic representation of Chordeumatida and Julida gonopods. Arrows indicate direction of sperm transfer.

a. Rings VI and VII of the male *Craspedosoma transsilvanicum* (Chordeumatidae), ventral view, illustrating position of anterior and posterior gonopods.

b. Anterior gonopods, aboral view. Sperm pass from the penes via vesicles at the base of the syncoxite to the syncoxal brushes. These enter the spermathecae during copulation. The clasping hook on the cheirite is homologous to the Spirostreptidae telopodite (see text, after Tadler 1993).

c. Diagrammatic representation of *Cylindroiulus boleti* (Julidae) gonopods, mesal view. Sperm transfer to the vulvae is accomplished by the distal ends of the posterior gonopods (after Tadler 1996).

(Haaker and Fuchs 1970). These have since been interpreted as stimulatory in the context of female choice (Eberhard 1985) and most recently as both direct and indirect sperm displacers (Tadler 1996; Tadler 1996 citing H. Enghoff pers. comm.).

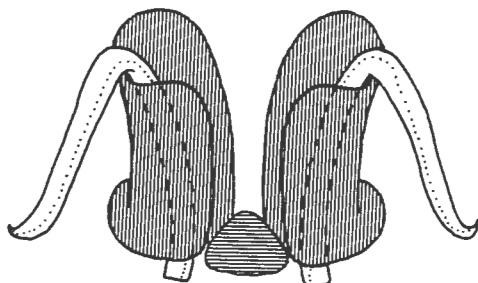


FIGURE 2.2 Diagrammatic representation of Spirostreptida gonopods. The coxite is folded to form a groove, the gonocoel, in which the telopodite is held. Gonopods are formed from the anterior legs only.

□ = telopodite; ▨ = coxite; ▩ = sternite.

2.1.3 Spirostreptida gonopods

In the Spirostreptida, gonopods are formed from the anterior legs only, except the Odontopygidae in which the sternite from the posterior legs remains (Attems 1928). They comprise three components, the coxite, the sternite and the telopodite (Fig. 2.2), the form and association of which are family-specific. In all three Spirostreptida families (see Appendix A), the coxite is folded to form a tube, the gonocoel, within which the telopodite is held. At the base of the coxite a gland known as the coxal ("*coxaldrüse*", Verhoef 1898) or prostate gland ("*gland prostatique*", Brölemann 1917) opens via a small protuberance into a canal, which runs along the length of the telopodite and allegedly functions to carry a secretion from the gland (Attems 1928). The telopodite canal is easily distinguished in fresh tissue but, as it is not a topographic feature, cannot be discerned under scanning electron microscopy (SEM).

Spirostreptida gonopods are inserted once only at the onset of copulation. Gonopod functional morphology is poorly understood. Of the three components that form the gonopods (the coxite, sternite and telopodite), only the telopodite moves during copulation via retraction and release of the "*anker*" (Krabbe 1982), a muscle complex situated at its base (Demange 1959). It has been suggested that sperm collect at the base of the coxites prior to gonopod insertion and are pumped into the vulvae through the telopodite canal (Demange 1959).

This chapter describes gonopod morphology for 26 Spirostreptida species, and functional morphology for five of these. Explanations for the evolution of Spirostreptida gonopods are considered.

2.1.4 Sperm morphology

Cognisance of sperm morphology is important because it can influence the mechanisms of competition both in terms of displacement (filamentous sperm may adhere to displacement devices more easily) and placement (placement is more critical for non-motile sperm because they cannot independently change their positioning within the sperm stores).

The ultrastructure of Diplopoda sperm from different orders is distinct (Jamieson 1987). The general evolutionary trend is towards simplicity and immotility (Warren 1934; Baccetti *et al.* 1979; Jamieson 1987), and all lack flagellae and/or motile processes (Baccetti 1970; Baccetti *et al.* 1979).

In the polyxenid *Polyxenus lagarus*, in which sperm transfer is indirect (Blower 1985), sperm are barrel shaped in the male reproductive tract and elongate into ribbon-shaped cells in the vulvae due to the acrosome reaction (Baccetti *et al.* 1974). Chilognaths have what have been termed "biscuit-shaped" sperm (Baccetti *et al.* 1979) that take the form of concavo-convex discs or triangles (see Warren 1934; Hortsman 1970; Jamieson 1987). With the exception of the Glomerida and Julida, Chilognath spermatozoa can pair up, in which case their concave surfaces are juxtaposed (Reger and Cooper 1968; Horstmann and Breucker 1969; Baccetti *et al.* 1979; Reger and Fitzgerald 1979). Paired sperm have been termed spermatophores (Horstmann and Breucker 1969). Pairing is known to persist in the female (Baccetti and Dallai 1978; Baccetti *et al.* 1979). The sperm of the Juliformia have bilayered acrosomes (Baccetti *et al.* 1979) and undergo an acrosome reaction involving the extrusion of a long filament just prior to fertilisation in the vulvae (Baccetti *et al.* 1977; Jamieson 1987). In the past, reacted acrosomes have been misidentified as flagellae (Gilson 1886; Oettinger 1909 cited in Reger and Fitzgerald 1979).

In spite of the belief that sperm morphology supports the current Diplopoda classification (Baccetti 1970; Jamieson 1987; Hopkin and Read 1992), sperm have not been considered as taxonomic characters, and a comparison of interspecific

morphology within families has not been provided for any Diplopod family. Here basic sperm morphology is described for 21 Spirostreptida species and the implications for ejaculate placement are discussed.

2.2 Materials and Methods

2.2.1 Preparation of gonopods for light microscopy (LM) and scanning electron microscopy (SEM).

Males were sacrificed in ethyl acetate killing jars and their gonopods were everted with forceps and removed. Gonopods to be used for observation under light microscopy were placed on glass slides and photographed with a Wild M400 microscope with camera back.

Gonopods used for scanning microscopy were thoroughly rinsed in physiological saline and fixed in 2.5% glutaraldehyde (pH 7.4 : phosphate-buffered saline) at 4°C for 24 hours. Secondary fixation in osmium tetroxide (2%) was followed by dehydration through a graded alcohol series (30% through absolute ethanol) and critical point drying. Samples were mounted on specimen stubs, sputter-coated with gold-palladium and examined under a Cambridge S200, a Leica 440 or a JEOL840 Scanning Electron Microscope.

Drawings of gonopods were produced using electron micrographs and photo micrographs with the aid of a Eiki 840 AXE Slide projector with built in screen. Drawings are stylised and are intended to portray gonopod morphology with respect to function.

2.2.2 Elucidation of gonopod functional morphology

Retraction and release of the "*anker*" (Krabbe 1982) at it's base causes the telopodite arm to perform a sequence of twists and turns that depend on the configuration of the telopodite arm and it's association with the coxite.

Functional morphology of the telopodites was elucidated in two ways.

a. Matings were generated in glass arenas (30 cm x 50 cm x 20 cm) containing equal numbers of males and females (each sex, $n = 10$), and upon formation, copula pairs were separated by carefully uncoiling the male from around the body of the female. When pairs are separated but their mouthparts are allowed to remain engaged, the male continues to retract and release the telopodites of his gonopods. These telopodite movements were observed and recorded.

b. The gonopods were removed and the telopodite was retracted manually by pulling gently at its base with forceps. Because the gonopods are non-inflatory chitinous structures, this manipulation simulates telopodite movement as effected by the male.

2.2.3 Collection and observation of spermatozoa

Sperm were collected with 10 μ l capillary tubes from either the everted gonopod coxites of males who were separated from females mid-copula, or the testes of dissected males. The contents of the tubes were smeared onto glass slides, mounted in insect saline and photographed with a Leica Lab K microscope with camera back, using both bright field and phase contrast microscopy. In total, the spermatozoa of nine Harpagophoridae, eight Spirostreptidae and three Odontopygidae species were examined.

2.3 Results

2.3.1 Gonopod morphology

Family HARPAGOPHORIDAE

In Harpagophoridae gonopods (Fig. 2.3), the gonocoel opens throughout its length on the lateral side of the coxite (Fig. 2.3b), or the basal part opens at the oral side and then runs obliquely to the lateral side (Fig. 2.3c, Attems 1928). The medial margin of the coxite usually bears one or two teeth on the aboral side. The telopodite (Fig. 2.3d) bends outwards laterally as it emerges from the coxite. It also typically bears one or two long femoral spines (Attems 1928), and divides into two branches at its tip. The first bears a plate that (usually) bears rigid comb-like teeth or spines (Attems 1928;

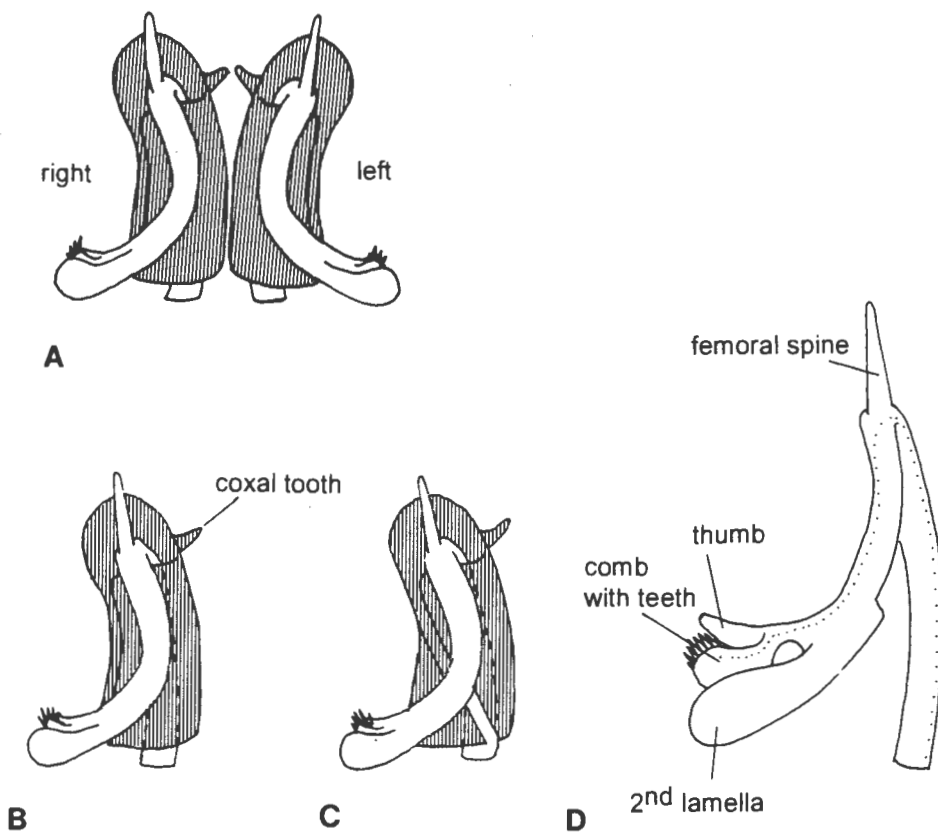


FIGURE 2.3 A. Generalised Harpagophoridae gonopod, aboral view, **B & C** . Right gonopod showing coxite variation, **D** . Generalised Harpagophoridae telopodite.

▨ = coxite; □ = telopodite; ▩ = telopodite canal

1937), and a lamella known as the thumb, through which the telopodite canal runs. The second branch of the telopodite is generally broadly rounded and is known as the second lamella (Fig. 2.3).

The gonopods of six Harpagophoridae species are described in Figures 2.4 - 2.9 and Plates 2.1 - 2.6.

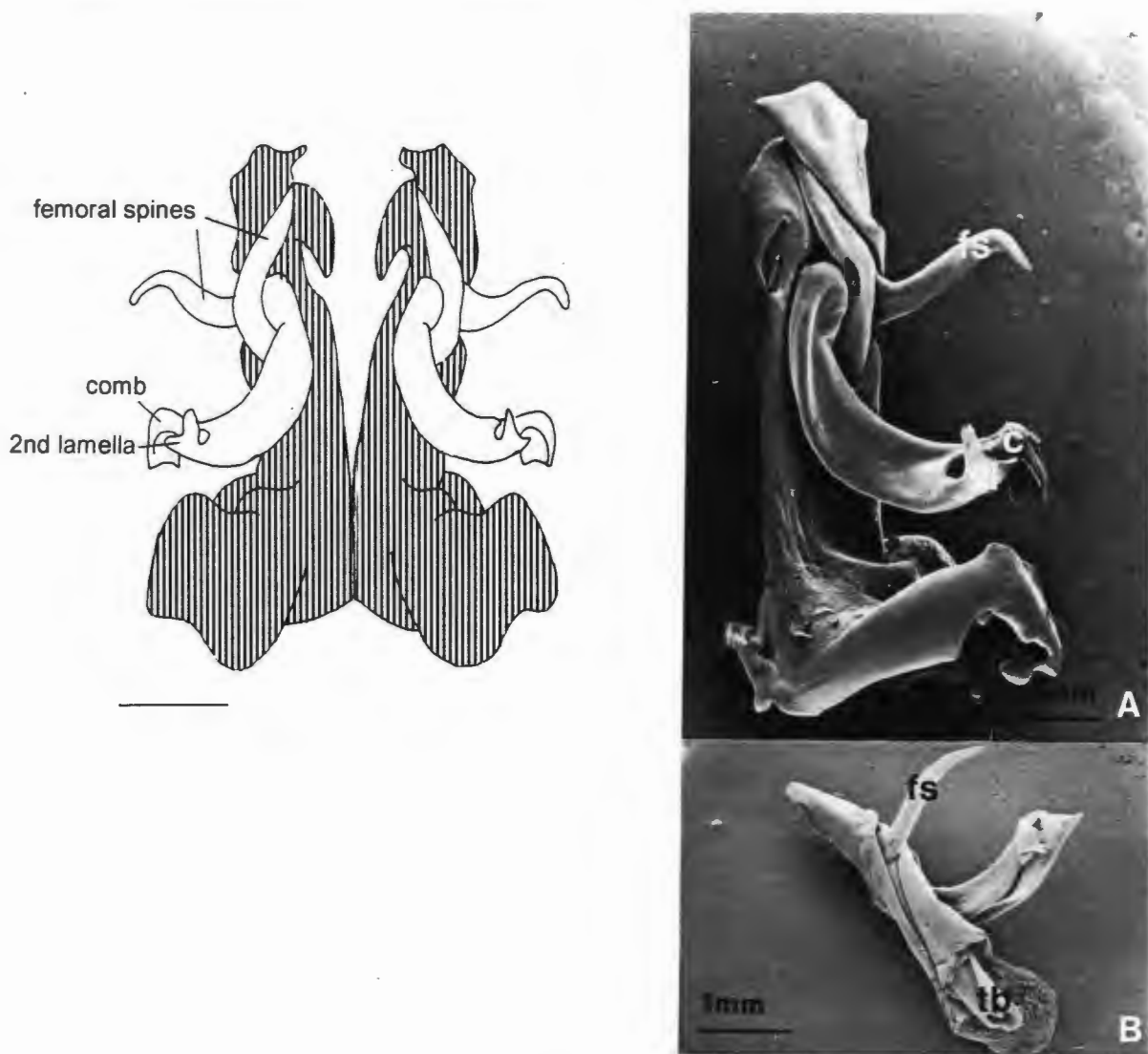


FIGURE 2.4 Aboral view of the gonopods of *Harpagophora levis* (Attems).
Scale bar = 1mm.

PLATE 2.1 A. Left gonopod, aboral view (SEM).
B. Right gonopod, lateral view (SEM).

Gonopods of *H. levis* bear two femoral spines on the telopodite that are nearly equal in length. One spine is straight and is directed distally, where it is partly concealed by the coxite (A). The other is free and points laterally. The telopodite terminates in a rounded comb, which bears several pointed spines, and a second lamellae. The thumb is absent. Note the hollow nature of the telopodite, as is visible at its base (B).

▣ = coxite; □ = telopodite, fs = femoral spine, c = comb, 2l = 2nd lamella, tb = telopodite base.

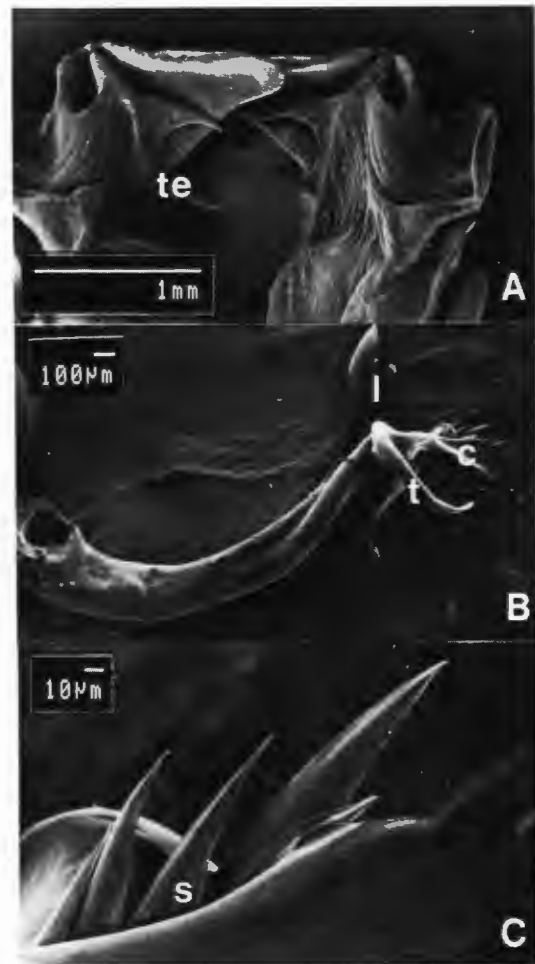
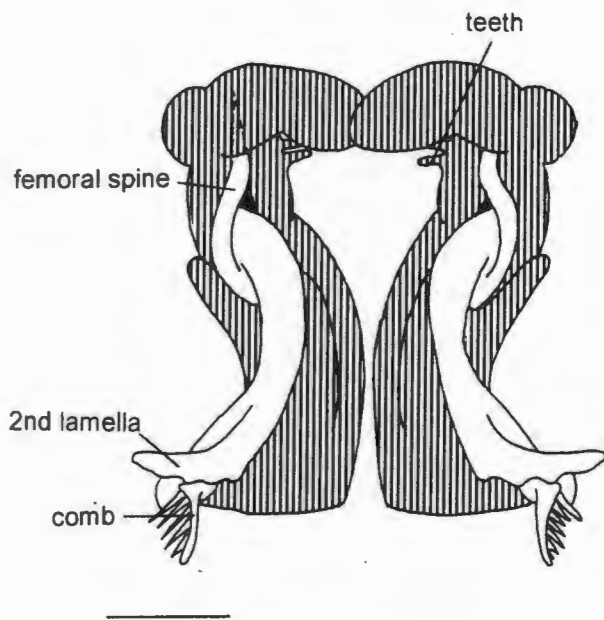


FIGURE 2.5 Aboral view of the gonopods of *Poratophilus (philoporatia) diplodontus* (Attems).
Scale bar = 1mm.

- PLATE 2.2**
- A. Distal gonopods, oral view (SEM).
 - B. Distal telopodite, lateral view (SEM).
 - c. Telopodite comb with spines (SEM).

P. diplodontus can be distinguished from other *Poratophilus* species by the distal medial lamella of the coxite which bears 2 pointed teeth (A) - all the other species bear only one. The femoral spine on the telopodite is directed distally and is partly concealed by the distal fold of the coxite. The distal tip of the telopodite (B) follows the generalised harpagophorid plan, and bears a comb lamella with a row of small spines (C). The thumb is slender and pointed and circles around the comb plate. (This differs from Attems 1928 in which the thumb is described as "cylindrical with the tip being blunt and curved into a semi-circle" and the comb bears a single long spine and a row of smaller ones, and may suggest two *P. diplodontus* sub species).

▣ = coxite; □ = telopodite, te = teeth, l = 2nd lamella, c = comb, t = thumb, s = spines.

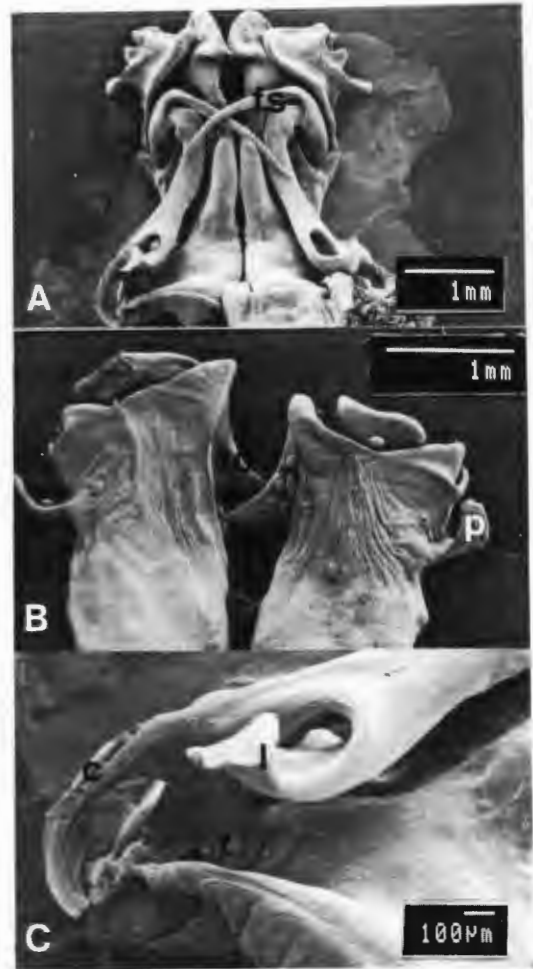
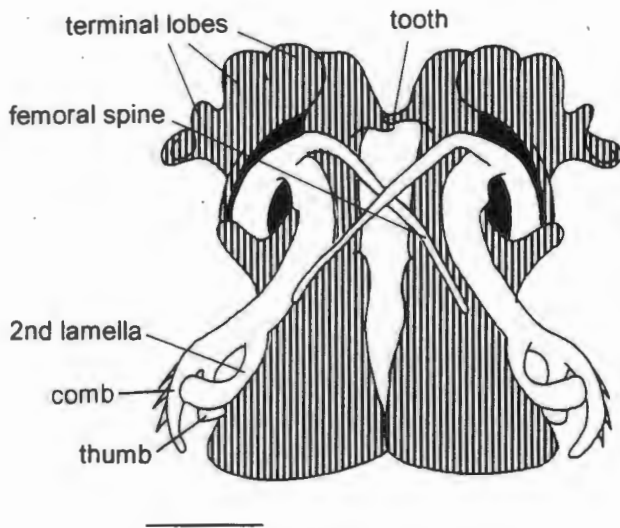


FIGURE 2.6 Aboral view of the gonopods of *Poratophilus (philoporatia) punctuatus* (Attems).
Scale bar = 1mm.

- PLATE 2.3**
- A. Aboral view of gonopods (SEM).
 - B. Distal gonopods, oral view (SEM).
 - c. Distal telopodite (SEM).

The coxite of each gonopod bears several terminal lobes and a single tooth on its medial margin (A) and a curved blunt process on its lateral margin (B). The femoral spine of the telopodite is free from the coxite and is long, slender, curved and transversely directed inwards. The distal end of the telopodite bears a comb with teeth that have a hyaline edge (Attems 1928), a thumb with several partly rounded lobes and a broad rounded second lamella (c)². ■ = coxite; □ = telopodite, fs = femoral spine, to = tooth, p = process, l = 2nd lamella, c = comb, te = teeth, t = thumb.

² The acariform mites present on the gonopods are probably of the genus *Caloglyphus* (Barnes 1980; Lawrence 1984). According to Lawrence (1939) they feed on seminal fluid.

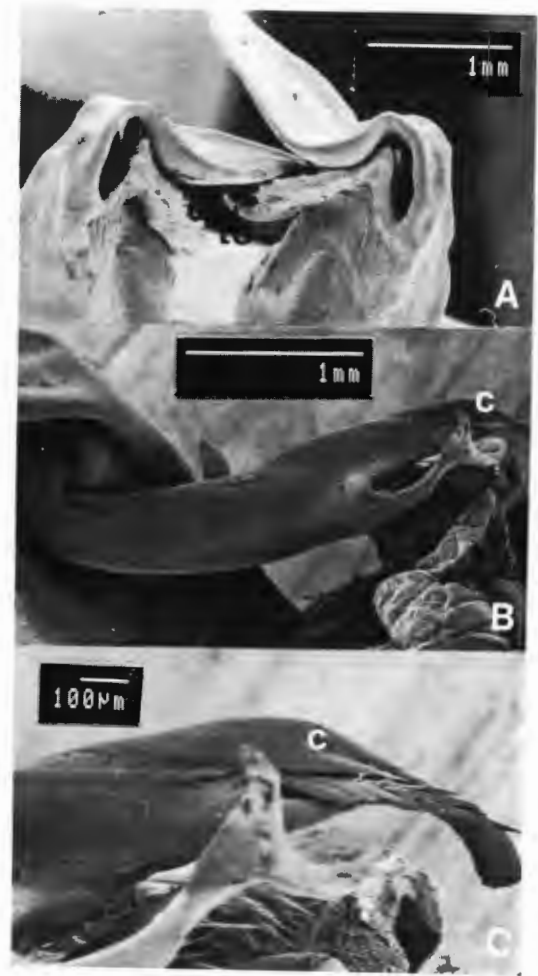
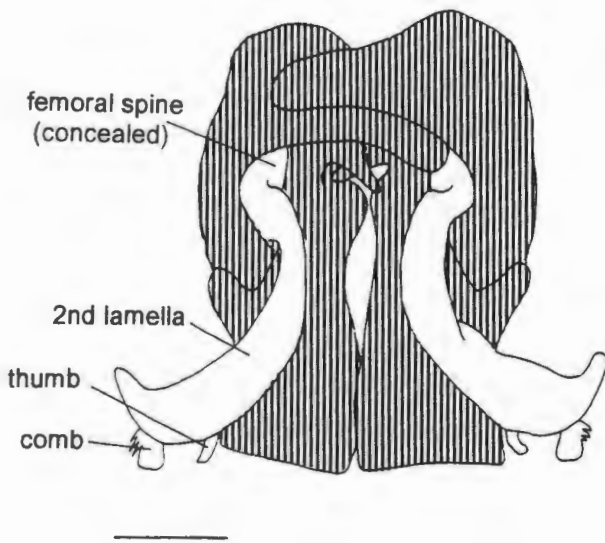


FIGURE 2.7 Aboral view of the gonopods of *Poratophilus* sp.1. Scale bar = 1mm.

- PLATE 2.4
- A. Aboral view of distal gonopods (SEM).
 - B. Telopodite (SEM).
 - C. Distal tip of telopodite (SEM).

The gonopod coxites are broad and rounded distally, and cross over at the medial margin. The medial margin bears a single large tooth and several smaller denticulate projections, that can be seen on the anterior lamella (A). The femoral spine of the telopodite is partly concealed beneath the distal coxite. The telopodite (B) bears a large rounded second lamella, a curved, tapering thumb and a comb with several sharp teeth that lie parallel to one another (C). The gonopods of these animals are similar to those of *P. similus* (Carl). They differ in the form of the back of the distal coxites which bear several parallel ridges in this species, and in body colouration (the body colouring of these millipedes is red-black, Carl's are described as yellow-black).

▣ = coxite; □ = telopodite, to = tooth, d = denticulate projections, c = comb.

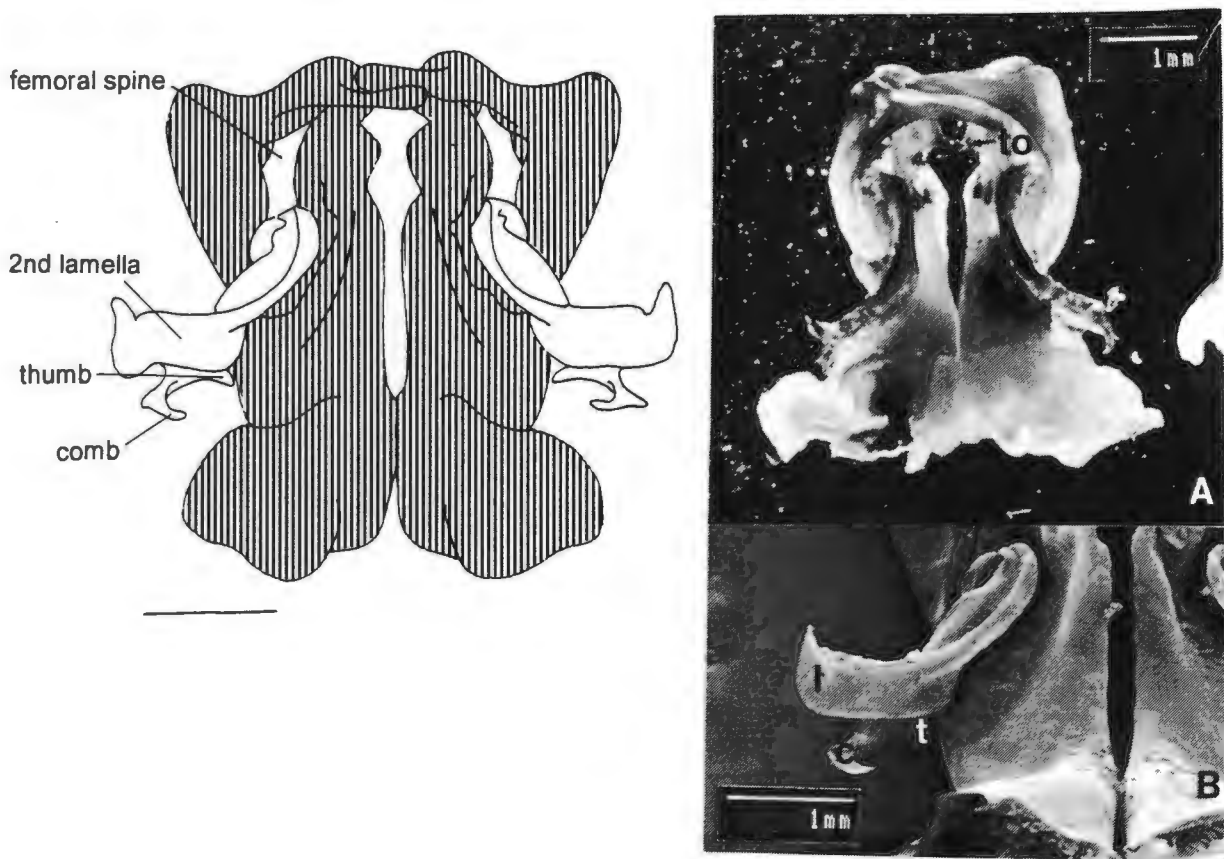


FIGURE 2.8 Aboral view of the gonopods of *Poratophilus* sp.2. Scale bar = 1mm.

PLATE 2.5 A. Aboral view of gonopods (LM)
B. Distal telopodite (SEM)

The terminal lamellae of the gonopod coxites each bear a horizontal ridge and cross over one another medially (A). A single tooth occurs on the medial coxite. The telopodite bears a single femoral spine that is upwardly directed and mostly concealed by the distal coxite (A). The distal end of the telopodite bears a rounded upwardly tilting second lamella, a flattened triangular thumb and a hooked comb that bears several parallel teeth (B).

▣ = coxite; □ = telopodite, to = tooth, l = second lamella, c = comb, t = thumb.

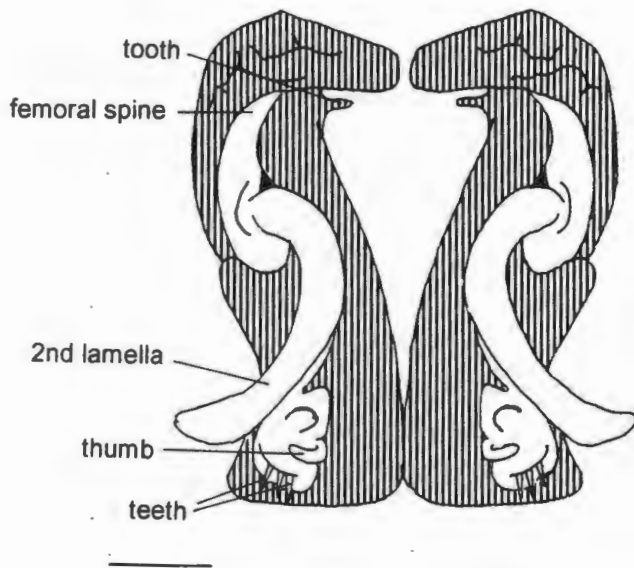


FIGURE 2.9 Aboral view of the gonopods of *Poratophilus* sp.3. Scale bar = 1mm.

PLATE 2.6 A. Aboral gonopods (SEM)
B. Distal tip of telopodite (SEM)

The distal gonopod coxites of this species are rounded and bear several shallow ridges, and the anterior coxite bears a single inwardly pointing spine (A). The telopodites are slender and extend into a tapering second lamella at their distal ends (B). The comb and thumb are lobed and the comb bears several thick parallel teeth.

▣ = coxite; □ = telopodite, to = tooth, l = 2nd lamella, c = comb, t = thumb..

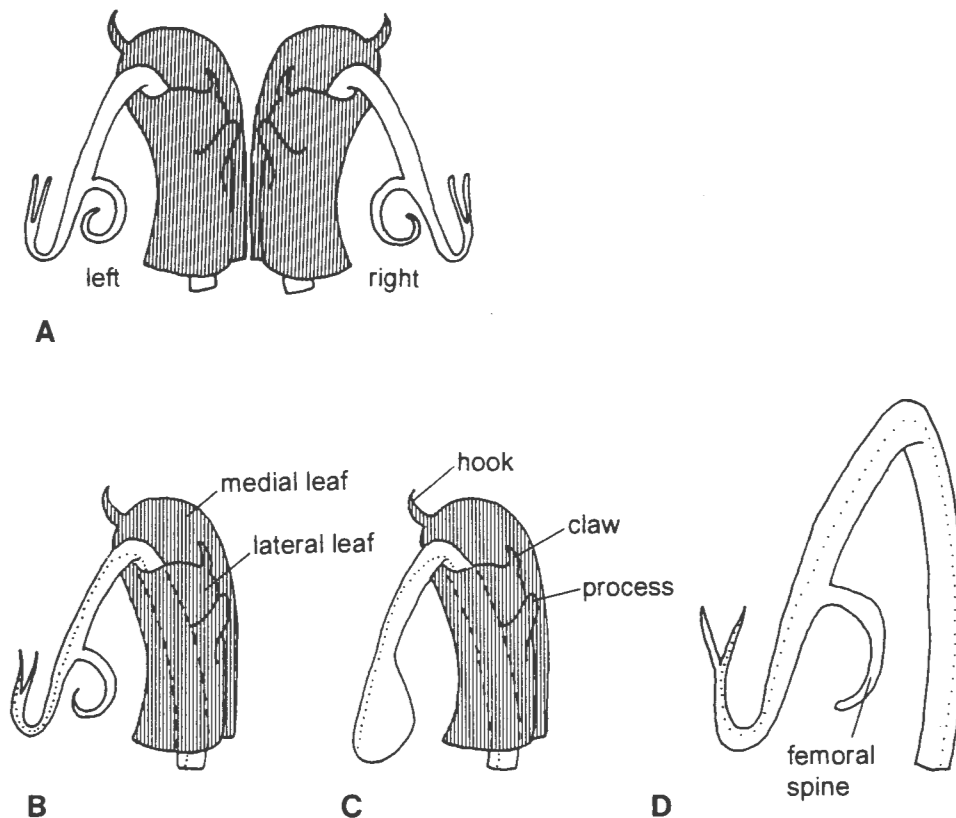


FIGURE 2.10 A. Generalised plan of the Spirostreptidae gonopod. Oral view., B & c. Left gonopod showing telopodite variation, D. Spirostreptidae telopodite. ▨ = coxite; □ = telopodite; ▩ = telopodite canal

Family SPIROSTREPTIDAE

In the Spirostreptidae, the gonocoel opening is medial and the fold at the lateral margin of the coxite forms a bridge over which the telopodite traverses. The coxite can bear several processes; a claw on the lateral leaf, and both a hook and a blunt process on the medial leaf. The Spirostreptidae also typically bear a region of spines on the distal oral coxite (Attems 1928; 1937). These vary in form from stout spines to hair-like bristles that are sometimes situated in pits or on papillae. Spirostreptidae telopodites are slender and bend out laterally when emerging from the gonocoel. Telopodites sometimes bifurcate medially giving rise to a femoral spine, and pointed processes (e.g. *Alloporus* spp.), or their distal ends take the form of rounded plates (e.g. *Orthoporoides* spp.) (Attems 1928; 1937) (Fig. 2.10).

The gonopods of thirteen Spirostreptidae species are described in Figures 2.11 - 2.23 and plates 2.7 - 2.19.

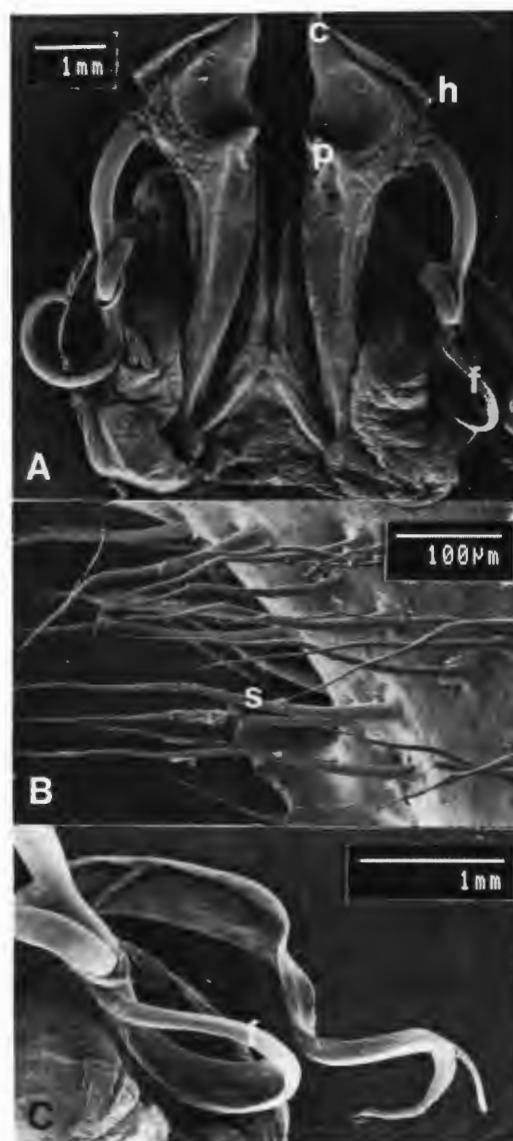
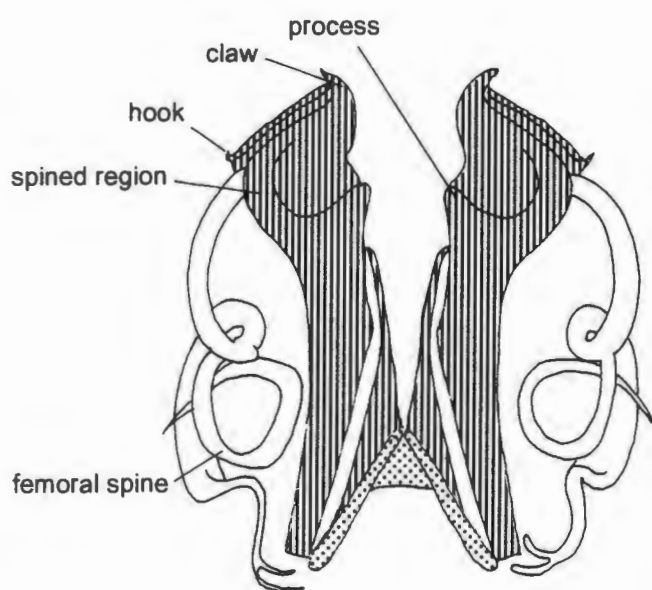


FIGURE 2.11 Oral view of the gonopods of *Alloporus bilobatus* (Schubart). Scale bar = 1mm.

PLATE 2.7 A. Oral view of gonopods (SEM), B. Spines of distal coxite (SEM)
c. Distal tip of telopodite (SEM)

As in all Spirostreptidae millipedes, the telopodites of the gonopods of *A. bilobatus* originate at the base and emerge from the gonocoel sleeve formed by the coxite. Laterally, the medial leaf of the gonopod coxite bears an inwardly turned curved hook (A). The lateral leaf terminates in a long curved claw which is black at the top and rises above the medial leaf. The lateral leaf also bears a process with a flat surface. Spines occur laterally on the distal lateral leaf and are long, thin and without papillae at their bases (B). The telopodite bears a femoral spine that describes a circle. The distal tip of the telopodite bears two processes, both of approximately equal length (C).

■ = coxite; □ = telopodite; c = claw, h = hook, p = process, f = femoral spine, s = spines

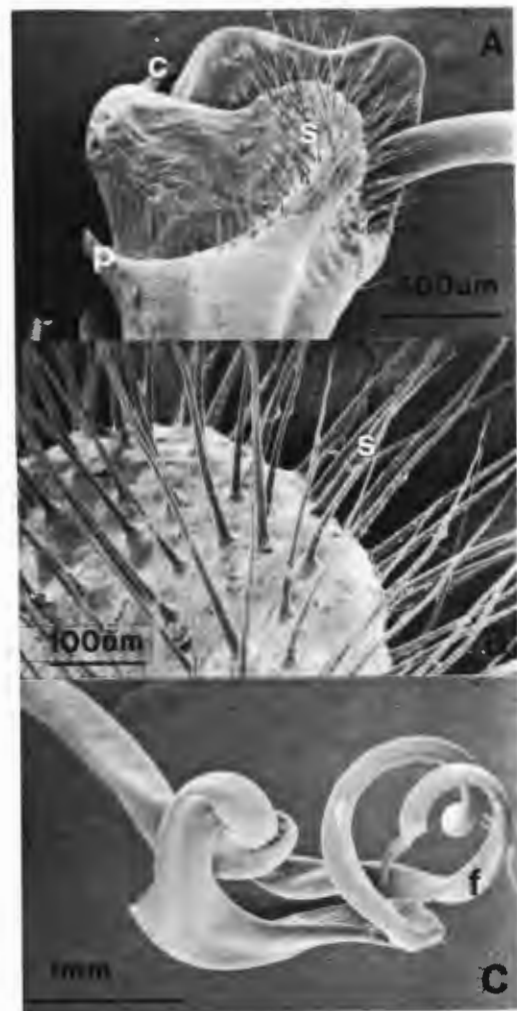
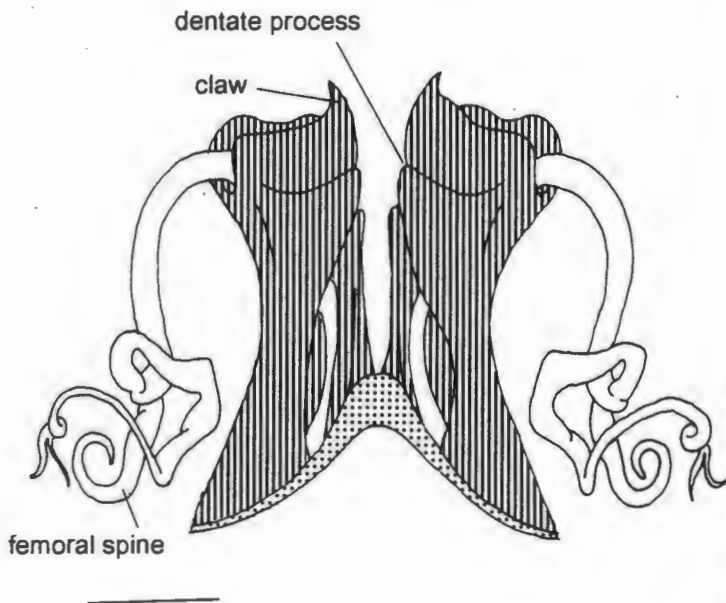


FIGURE 2.12 Oral view of the gonopods of *Alloporus castanaes* (Attems). Scale bar = 1mm.

- PLATE 2.8**
- A. Distal oral coxite (SEM)
 - B. Detail of spined region of oral coxite (SEM)
 - C. Distal tip of telopodite (SEM)

The medial leaf of the gonopod coxite is rounded distally and bears a small black hook on its lateral margin (that cannot be seen because it curves back behind the leaf) (A). The lateral leaf of the coxite bears a dentate process on its inner border and a long terminal claw (A). On the terminal lateral border, the coxite bears a region of spines ($\pm 250\mu\text{m}$ long) that are situated in shallow pits (B). The telopodite bifurcates medially giving rise to a femoral spine which tapers at its distal end, and an arm that bears the telopodite canal. The latter arm forks distally, the longer branch carrying the telopodite canal (C).

▨ = coxite; □ = telopodite; c = coxite, p = process, s = spines, f = femoral spine

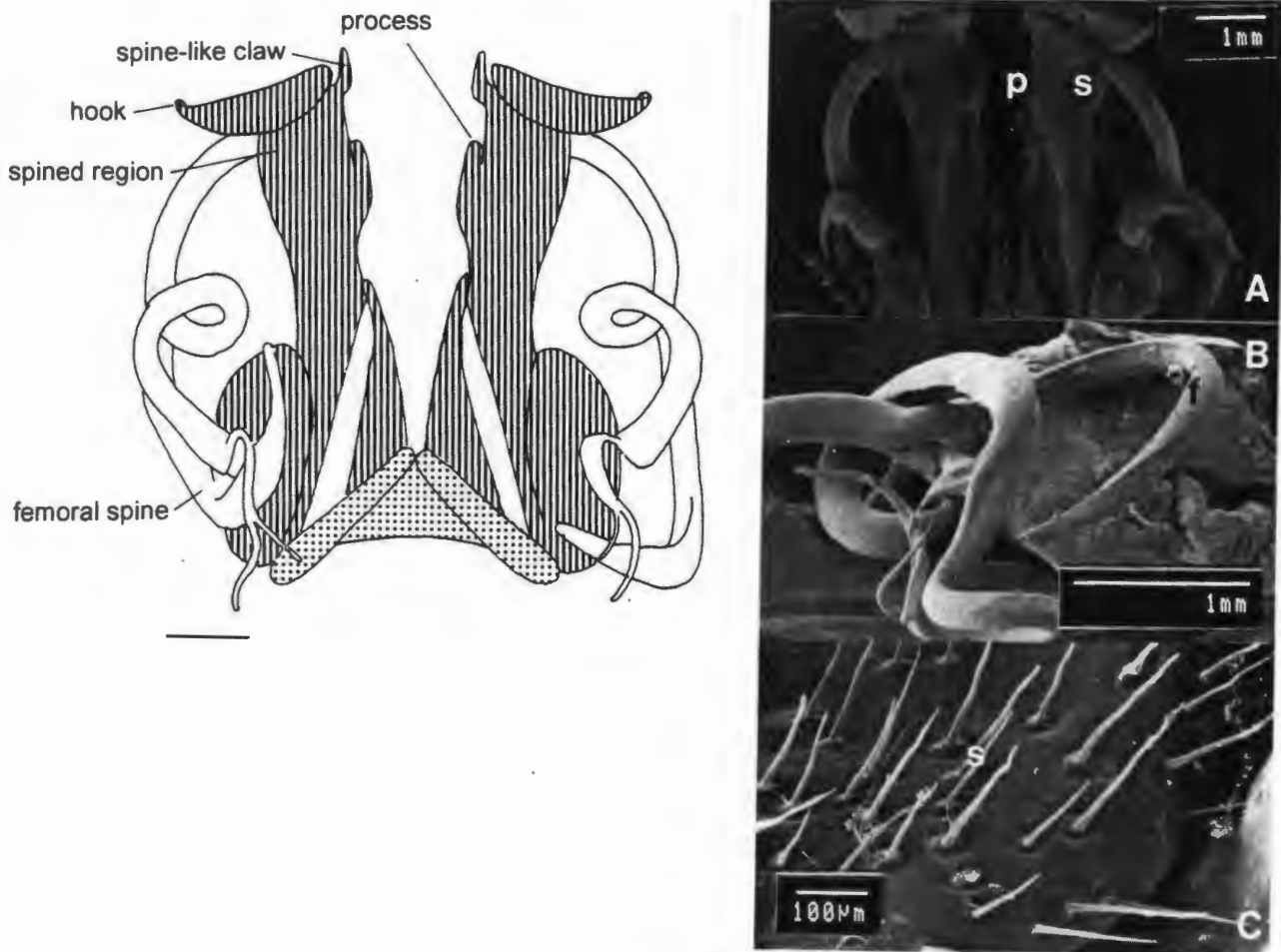


FIGURE 2.13 Oral view of the gonopods of *Alloporus falcatus* (Attems). Scale bar = 1mm.

- PLATE 2.9**
- A. Oral gonopods (SEM).
 - B. Distal telopodite (SEM).
 - C. Spined region on distal oral coxite (SEM).

Gonopods of *A. falcatus* bear a small hook on the lateral extremity of the medial leaf, which is drawn out to form a cone (A). The lateral leaf does not bear an enlarged distal claw but instead terminates with a thin spine (A). The telopodite bears a large tapering femoral spine that does not describe a circle (B) (compare to *A. bilobatus*, Fig. 2.11). An extensive area of spines ($\pm 150\mu\text{m}$ long) occurs down the front of the coxite. Spines are situated in shallow pits and on papillae (C).

▣ = coxite; □ = telopodite; p = process, f = femoral spine, s = spines

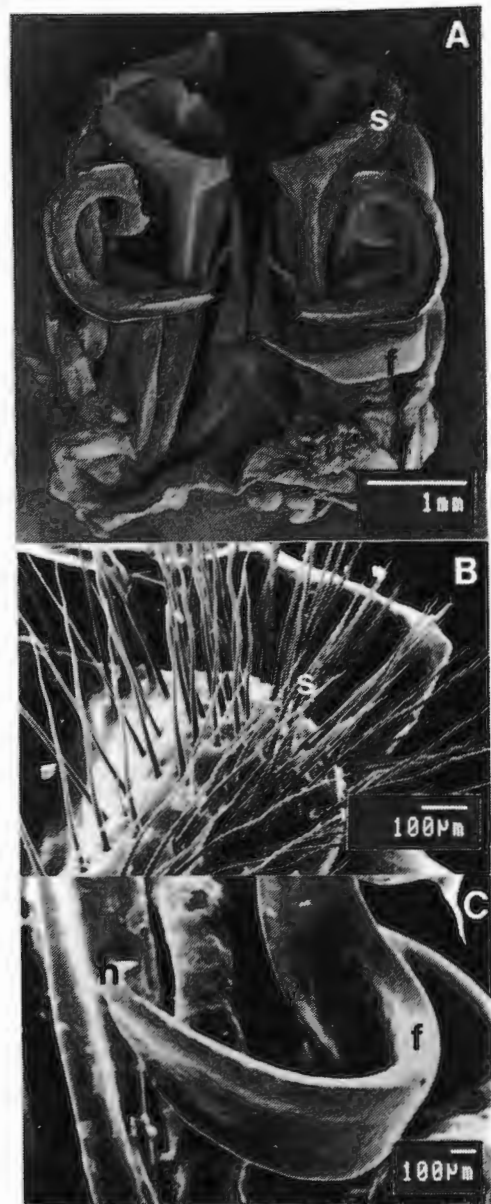
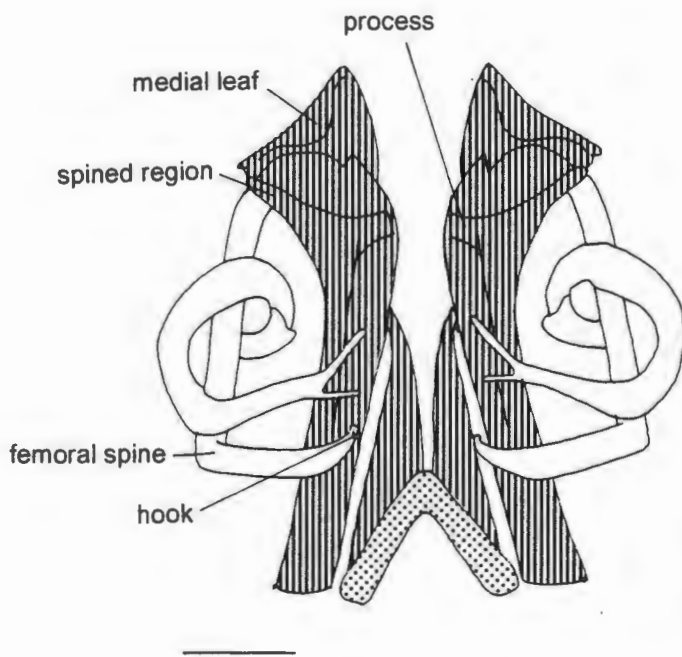


FIGURE 2.14 Oral view of the gonopods of *Alloporus flavifilis* (Peters). Scale bar = 1mm.

- PLATE 2.10
- A. Gonopods of *A. flavifilis*, oral view (SEM).
 - B. Distal oral coxite showing spined region (SEM).
 - C. Gonopod telopodite (SEM).

The medial leaf of the gonopods does not bear a hook and curves over the lateral leaf distally (A). On the lateral leaf there is a flattened medial process and a region of long spines ($\pm 300\mu\text{m}$ long), some of which occur in small pits (B). The telopodite arm that bears the canal bifurcates close to its tip, with one branch considerably shorter than the other. The femoral spine is broad and terminates in a hook (C).

▣ = coxite; □ = telopodite; f = femoral spine, s = spines, h = hook

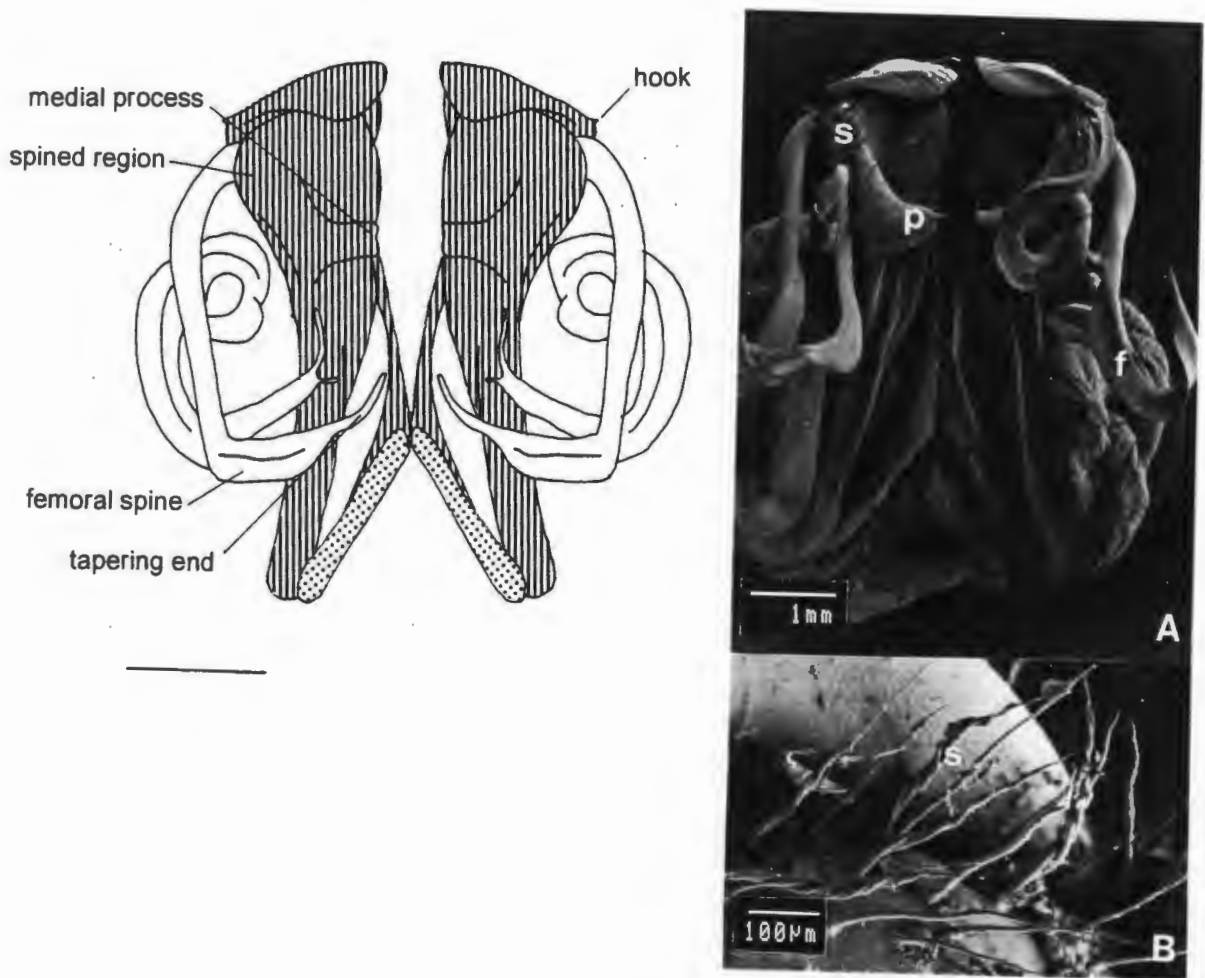


FIGURE 2.15 Gonopods of *Alloporus flavifilis* subsp. n., oral view. Scale bar = 1mm.

PLATE 2.11 A. Oral view of *A. flavifilis* subsp. gonopods (SEM)
B. Detail of spined region on distal coxite (SEM)

Gonopods of *A. flavifilis* subsp. differ from those of *A. flavifilis* in that the medial process on the lateral leaf is more distinct and terminates in a rounded black tip (A). Bristles occur on the medial side of the distal lateral coxite leaf and are $\pm 350\mu\text{m}$ long (B). The telopodite closely resembles that of *A. flavifilis*. It forks at its distal end and terminates in two branches of unequal length. The femoral spine is broad and flattened and tapers to a point, rather than ending in a hook as in of *A. flavifilis* (Peters).

▣ = coxite; □ = telopodite; s = spines, p = process, f = femoral spine

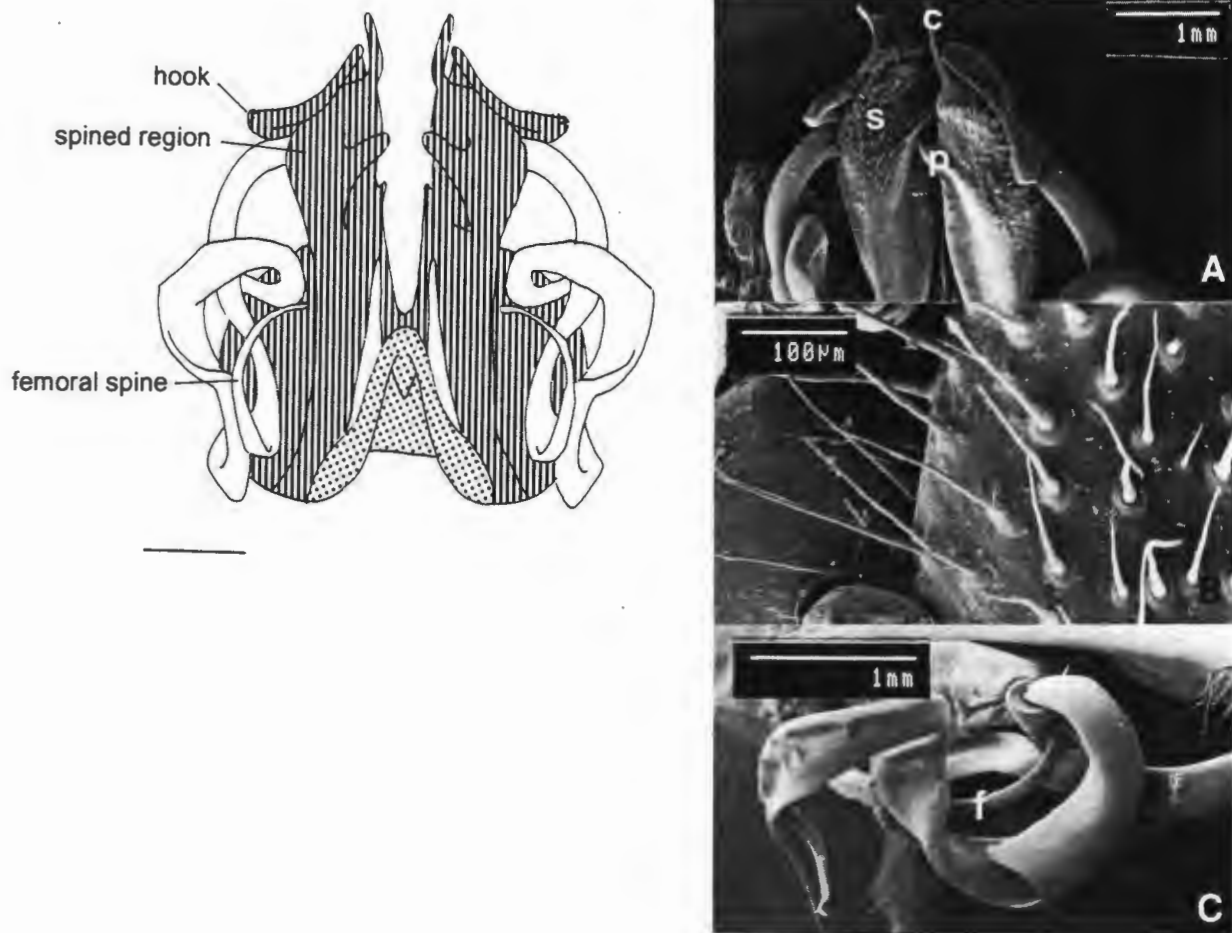


FIGURE 2.16 Oral view of the gonopods of *Alloporus levigatus* (Attems). Scale bar = 1mm.

- PLATE 2.12
- A. Distal oral gonopod of *A. levigatus* (SEM).
 - B. Spined region on distal oral coxite (SEM).
 - c. Gonopod telopodite

The pointed process on the inner side of the lateral leaf of each gonopod bears several teeth, and the lateral leaf terminates in a long pointed black claw (A). Spines (100-150 μ m long) cover the distal part of the oral coxite extending to the base of this claw, but not as far as the medial margin. They are situated on small papillae, and not in pits (B). The medial leaf of the coxite bears a small hook at the end of its lateral border. The telopodite bifurcates medially and bears a femoral spine that tapers and describes more than a complete circle, and an arm that bears the telopodite canal which forks distally (C).

▣ = coxite; □ = telopodite; c = claw, s = spines, p = process, f = femoral spine.

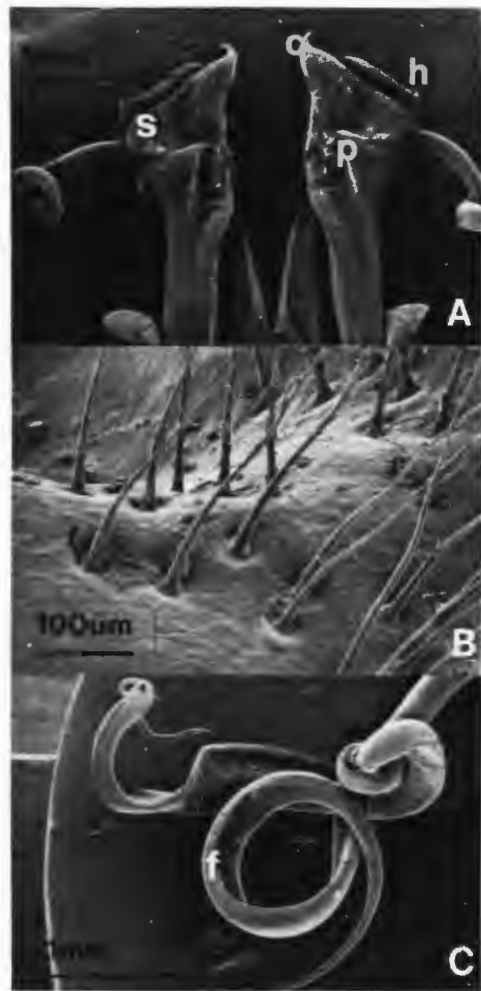
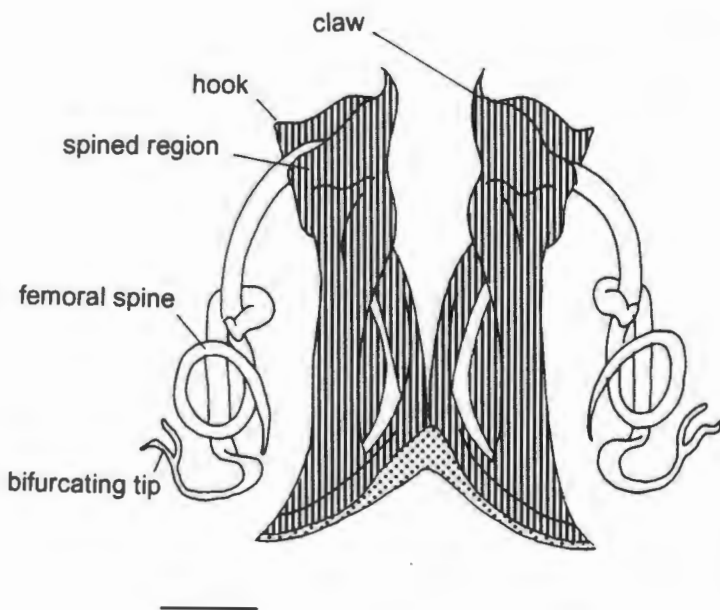


FIGURE 2.17 Oral view of the gonopods of *Alloporus rugifrons* subsp. (Attems). Scale bar = 1mm.

- PLATE 2.13 A. Distal gonopods, oral view (SEM).
 B. Detail of spined region on distal coxite (SEM).
 c. Distal telopodite (SEM).

The medial leaf of the gonopod coxite bears a small lateral hook. The lateral leaf bears a claw that extends beyond the medial leaf, and a medial process that is not well defined (vs *A. rugifrons* in which it is well defined) (A). Spines occur laterally on the distal oral coxite (B). The femoral spine describes a complete circle and terminates in a slender point (c) (In *A. rugifrons* a complete circle is not described).

▣ = coxite; □ = telopodite; c - claw, h = hook, p = process, s = spines, f = femoral spine.

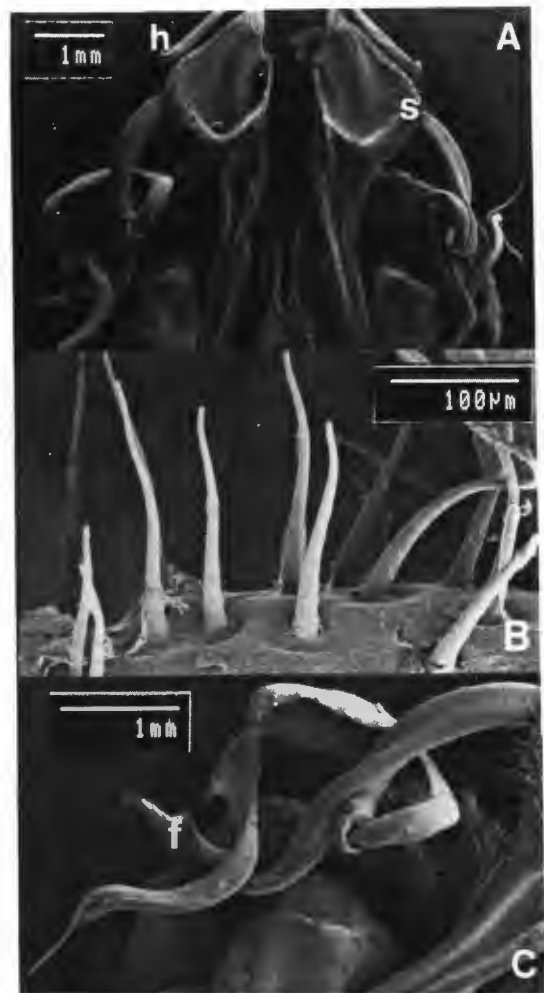
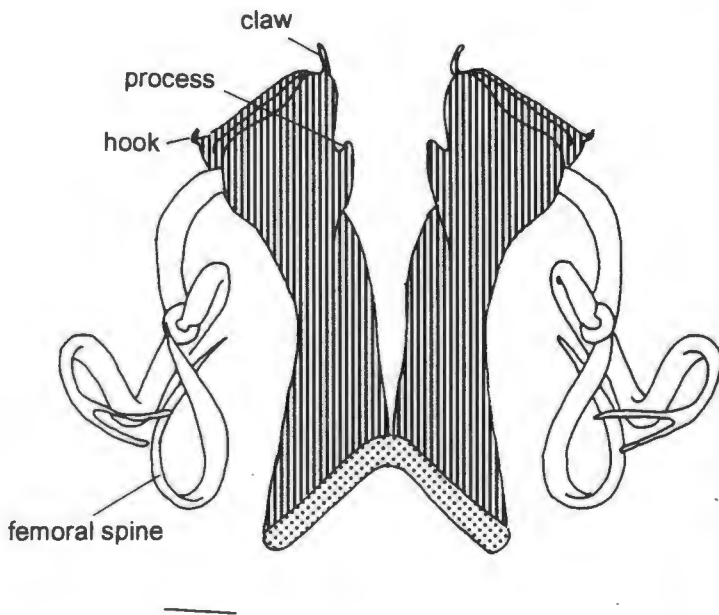


FIGURE 2.18 Oral view of the gonopods of *Alloporus transvaalensis* (Schubart). Scale bar = 1mm.

- PLATE 2.14**
- A. Oral view of gonopods (SEM).
 - B. Detail of spines on lateral distal coxite (SEM).
 - C. Distal tip of telopodite (SEM).

Each gonopod bears a small lateral hook on the medial leaf of the coxite (A). The lateral leaf bears a small claw that terminates in a spine, and a spined region on its lateral border. Spines are stout and short and are situated in pits (B). The telopodite terminates in two processes of equal length and bears a straight femoral spine that twists at its end (C).

▣ = coxite; □ = telopodite; h = hook, s = spines, f = femoral spine.

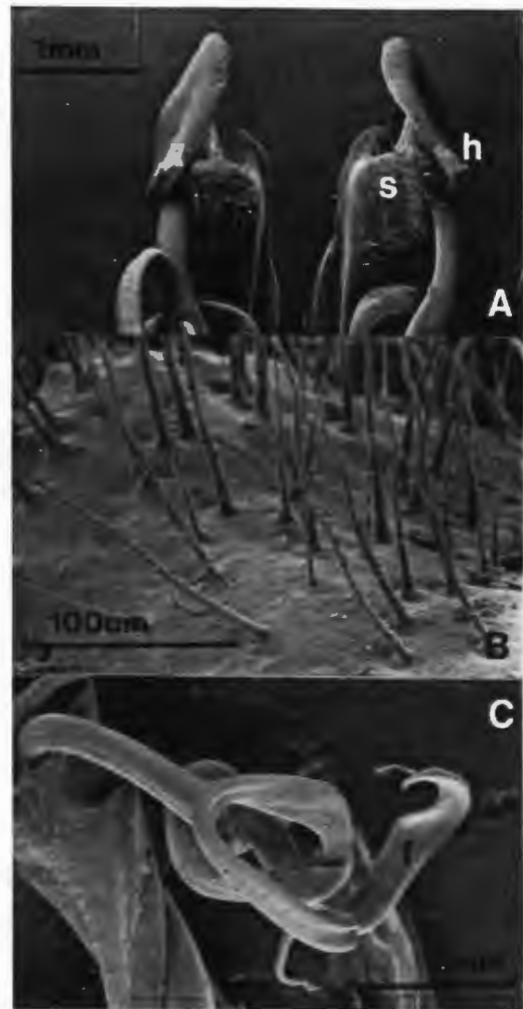
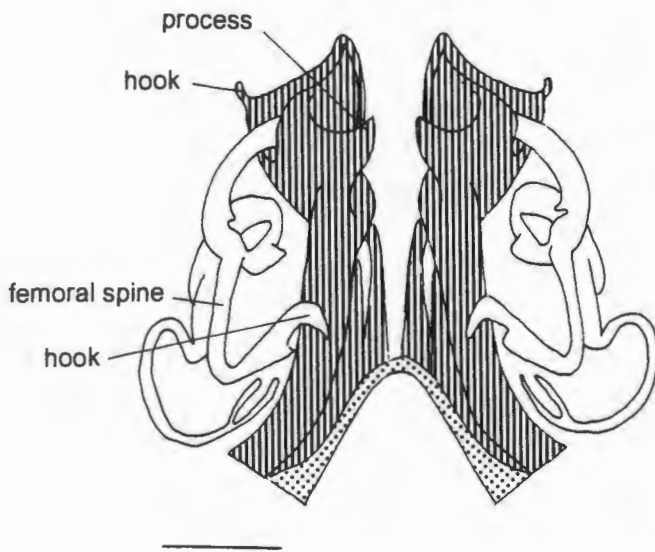


FIGURE 2.19 Oral view of the gonopods of *Alloporus uncinatus* (Attems). Scale bar = 1mm.

- PLATE 2.15
- A. Distal oral coxites (SEM).
 - B. Detail of spined region on distal oral coxites (SEM).
 - c. Distal gonopod telopodite (SEM).

The telopodites of the gonopods of *A. uncinatus* originate at the base and emerge from the gonocoel sleeve formed by the coxite. The coxite bears a black hook on its lateral side at the tip of the medial leaf (A) and a region of dense spines orally on the distal coxite surface. Spines are approximately 250µm long and some are situated in shallow pits (B). The lateral leaf of the coxite bears a blunt dentate process and does not end in a terminal spine or claw. The telopodite bifurcates shortly after emergence from the coxite. The thinner branch, the femoral spine, bears a characteristic flattened hook at its distal end. The thicker branch bears the telopodite canal, and forks at its distal end (C).

▣ = coxite; □ = telopodite; h = hook, s = spines, f = femoral spine.

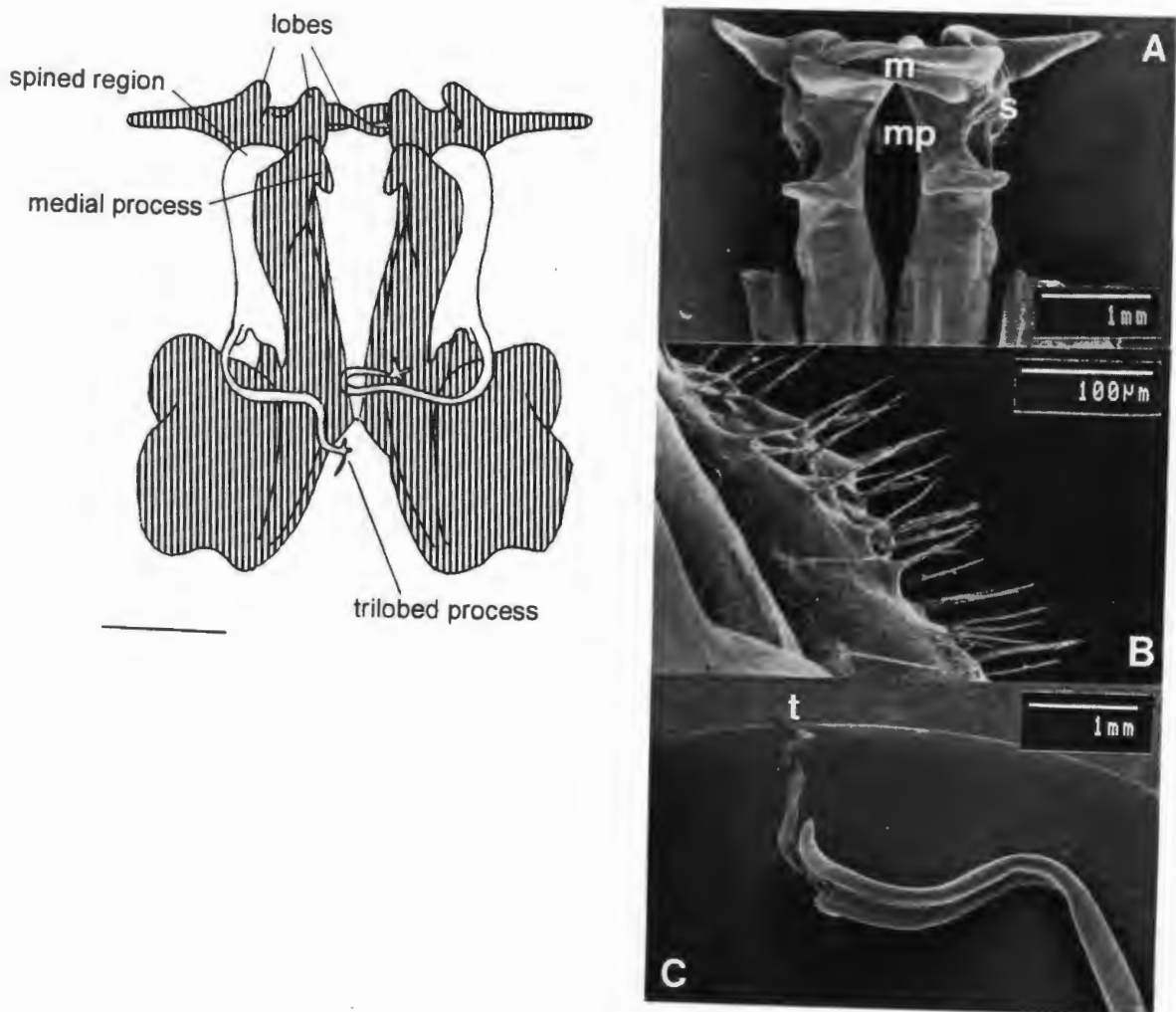


FIGURE 2.20 Oral view of the gonopods of *Bicoxidens matabele* (Schubart). Scale bar = 1mm.

- PLATE 2.16**
- A. Aboral view of distal gonopod coxites (SEM).
 - B. Detail of spined region on lateral oral coxite (SEM).
 - C. Distal telopodite (SEM).

The medial leaf of each gonopod bears three lobes. Two are distal, one thick and rounded and one slender and inwardly directed, and one is medial (A). The medial lobes are inwardly directed and cross over one another (A). The lateral leaf bears an area of spines $\pm 180\text{-}200\mu\text{m}$ long that are on small papillae (B). The telopodite is slender and does not bear a femoral spine. A single short spine splits off from it approximately two-thirds of the way down, from which point the telopodite is considerably thinner and terminates in a small trilobed process.

▣ = coxite; □ = telopodite; mp = medial process, m = medial lobes, s = spines, t = trilobed process.

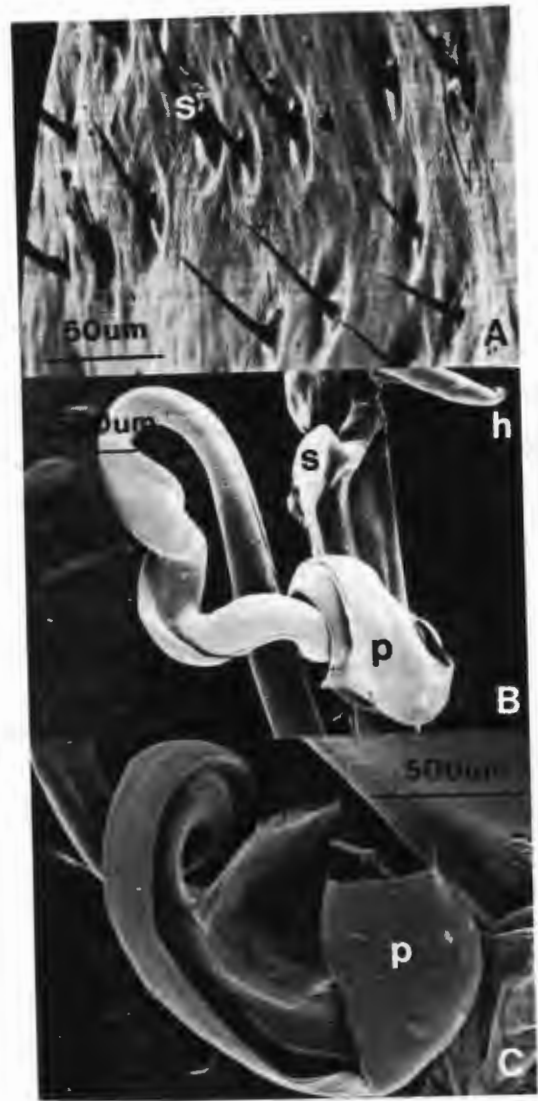
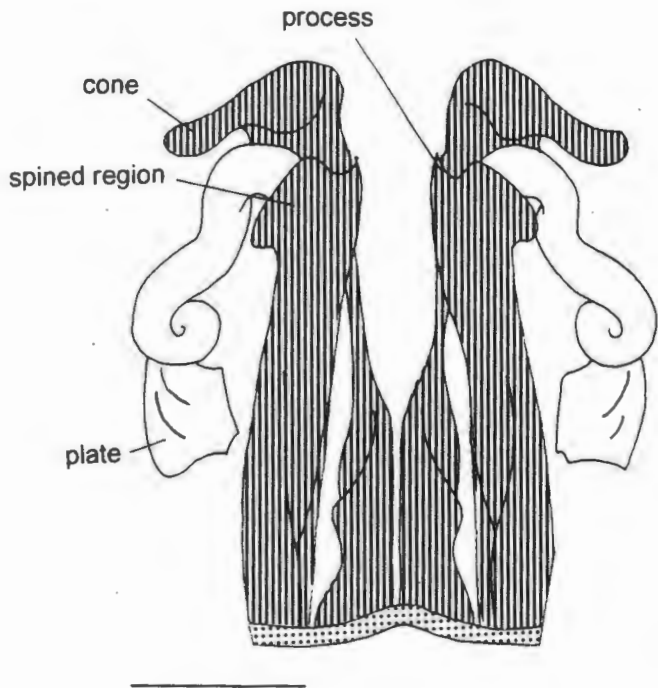


FIGURE 2.21 Oral view of the gonopods of *Orthoporooides pyrhocephalus* (Koch). Scale bar = 1mm.

- PLATE 2.17**
- A. Detail of spined region on distal oral coxites (SEM).
 - B. Gonopod telopodite (SEM).
 - C. Detail of distal end of telopodite (SEM).

A backwardly-inclined lateral cone that terminates in a hook occurs distally on the medial leaf of the coxite (B). The lateral leaf of the coxite terminates in a rounded disc immediately below the level of the medial leaf's cone, and forms a blunt process that curls around the medial leaf. An area of short, stout pitted spines ($\pm 50\mu\text{m}$) occurs on the distal lateral leaf (A). The telopodite emerges from the gonocoel as a slender shaft that describes three turns and terminates in a broad plate (B, C). The telopodite canal follows the course of the telopodite as a narrow tube below its surface, and terminates as a small opening on the trailing edge of the plate.

▨ = coxite; □ = telopodite; s = spines, h = hook, p = plate.

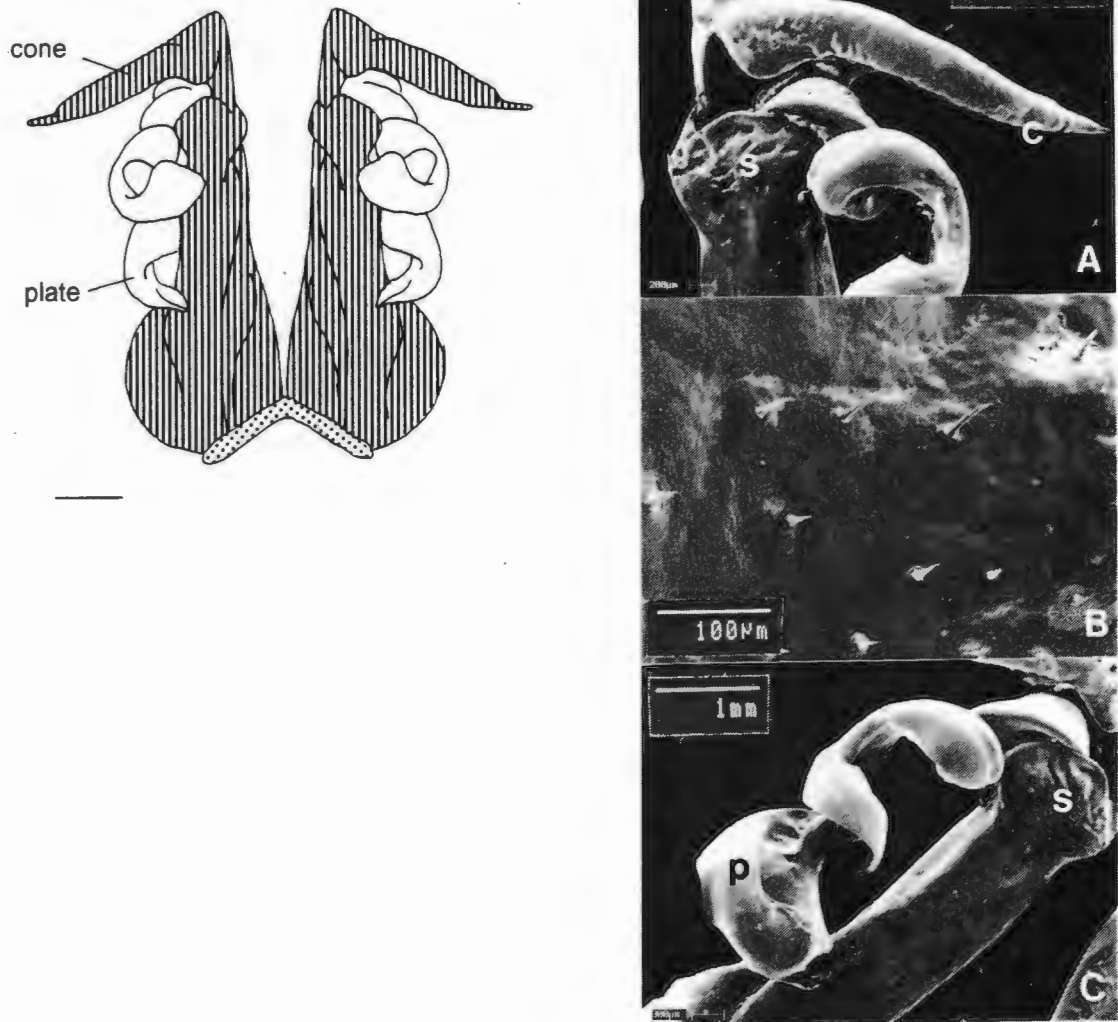


FIGURE 2.22 Oral view of the gonopods of *Orthoporoides tabulinus* (Attems). Scale bar = 1mm.

- PLATE 2.18**
- A. Detail of cone at tip of gonopod coxites (SEM).
 - B. Detail of spined region on distal oral coxite (SEM).
 - c. Distal telopodite (SEM).

The cone of the medial leaf of each gonopod tapers into a point, and does not bear an overturned hook (as in *O. pyrhocephalus*) (A). Distally the lateral leaf of the coxite bears a field of small spines that are not pitted (B). The telopodite is similar to that of *O. pyrhocephalus* and describes two turns before terminating in a broad plate (c).

▣ = coxite; □ = telopodite; c = cone, s = spines, p = plate

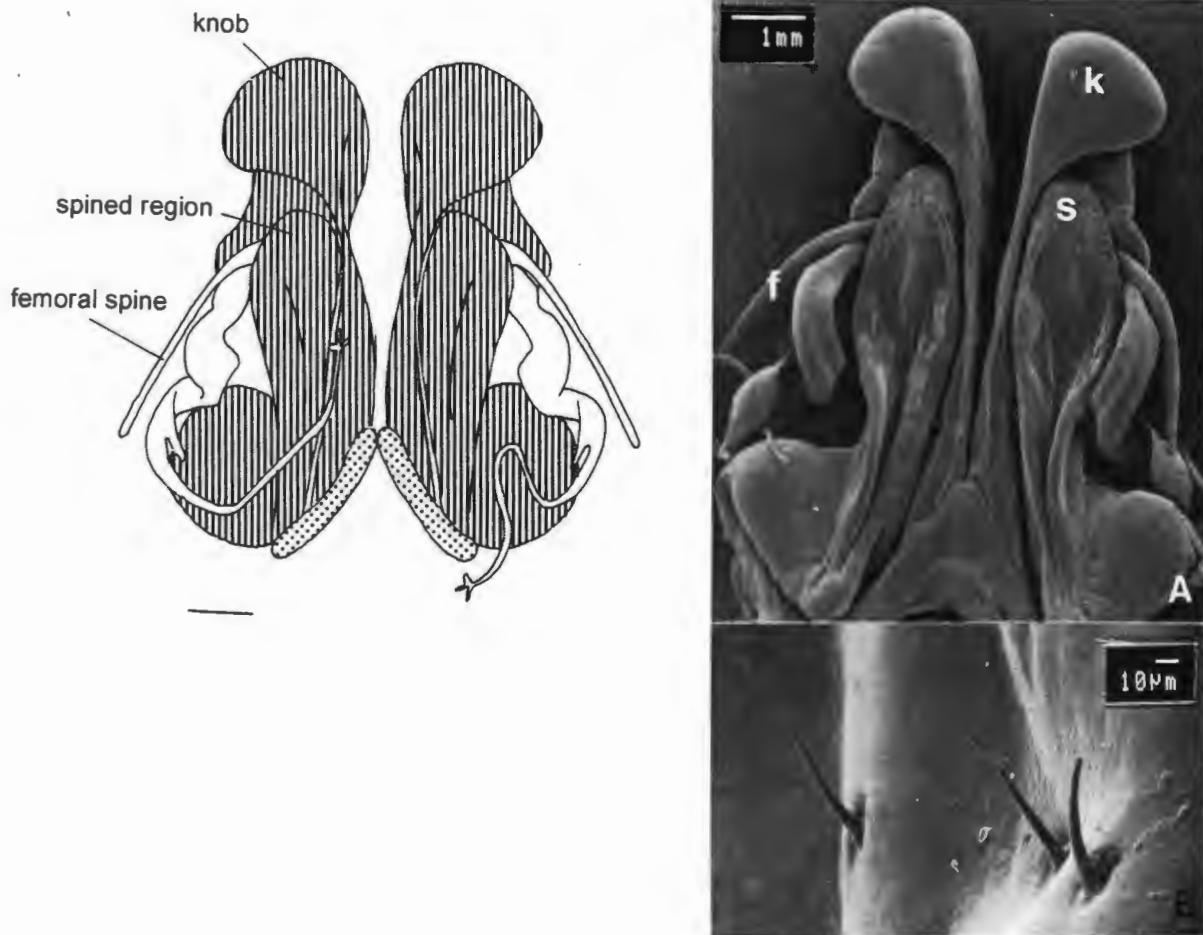


FIGURE 2.23 Oral view of the gonopods of *Triaenostreptus unciger* (Attems) Scale bar = 1mm.

PLATE 2.19 A. Gonopods of *T. unciger*, oral view (SEM).
B. Detail of spined region on oral coxite (SEM).

The tip of the medial leaf is bent outwards, forming a thick knob (A) that is easily distinguished in fresh tissue as a pale yellow structure. The tip of the lateral leaf is rounded and covered with spines $\pm 120\mu\text{m}$ in length (B). The telopodite is broad and performs two twists before splitting at its end into two processes, one of which is much longer than other. The femoral spine of the telopodite is long and straight and splits from the telopodite before it emerges from the gonocoel (A).

▣ = coxite; □ = telopodite; k = knob, f = femoral spine, s = spines.

Family ODONTOPYGIDAE

In the southern African *Odontopygidae*³ the telopodite originates at the base of the coxite but, unlike the *Harpagophoridae* and *Spirostreptidae*, is held loosely within a shallow gonocoel that extends only part of the way up the coxite. Telopodites are proportionally larger than those of the *Harpagophoridae* and the *Spirostreptidae* and upon leaving the gonocoel, they twist about themselves and bend medially. Telopodites bifurcate medially, bearing a whip-like process (a derivative of the tibia) that houses the telopodite canal, a broad plate (a derivative of the tarsus) (Attems 1928, p323) and sometimes one or two femoral spines.

Whereas in the *Harpagophoridae* and *Spirostreptidae*, the second pair of legs is completely absent, in the *Odontopygidae* the sternite of the second pair of legs remains (Brölemann 1917, Attems 1928) (Fig. 2.24).

The gonopods of seven *Odontopygidae* species are described in Figures 2.25 - 2.31 and Plates 2.20 - 2.26.

³ Of the three *Odontopygid* subfamilies, only the *Odontopyginae* are represented in southern Africa (Attems 1928). The above gonopod description is particular to this subfamily.

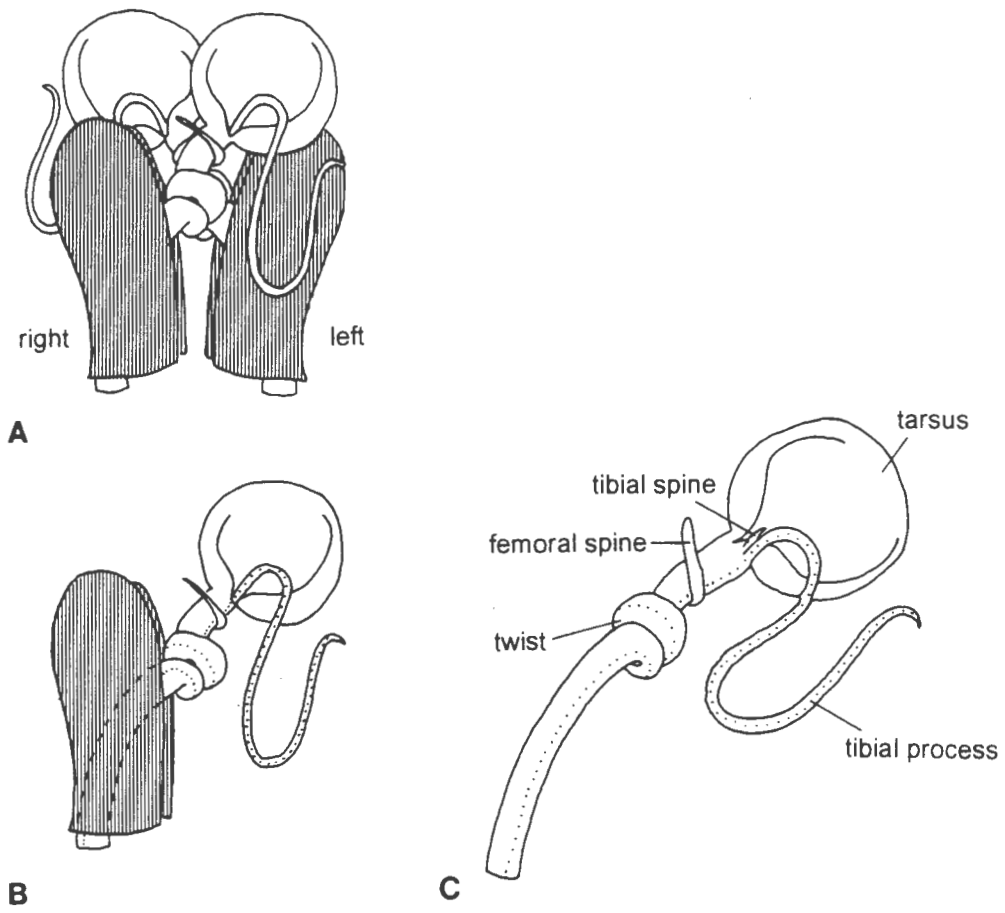


FIGURE 2.24 A. Generalised plan of the Odontopygidae gonopod. Aboral view, B. Right gonopod, C. Odontopygidae telopodite. \blacksquare = coxite; \square = telopodite; \square = telopodite canal

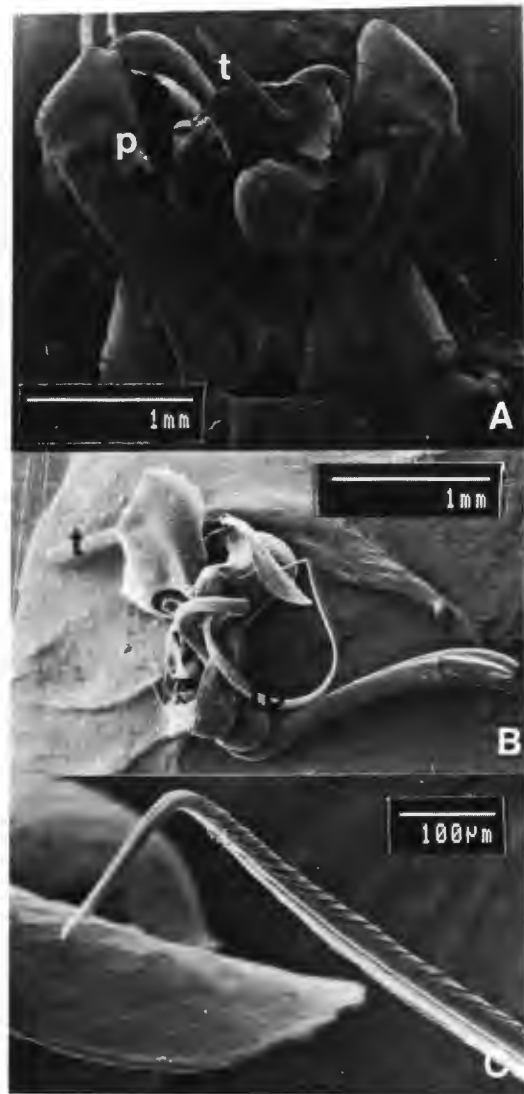
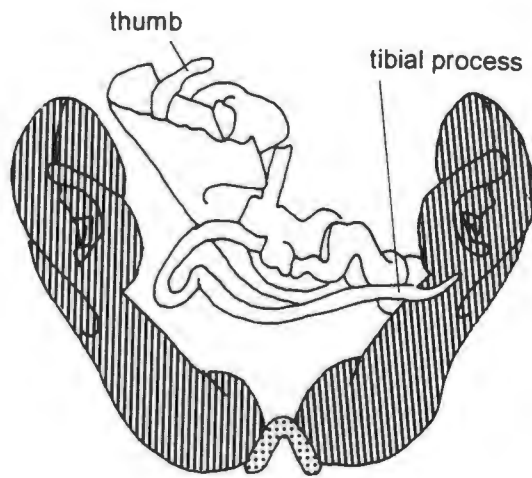


FIGURE 2.25 Oral view of the gonopods of *Chaleponcus digitatus* (Kraus). (The left telopodite is not drawn). Scale bar = 1mm.

PLATE 2.20 A. Aboral view of *C. digitatus* gonopods (SEM).
 B. Telopodite, removed from gonocoel of coxite (SEM).
 c. Distal telopodite (SEM).

The coxite of the gonopods is rounded distally (A) and bears a curved medial process that is visible aborally. The telopodite bears a long tibial process (B) that terminates with several overlapping plates and a sharp point (C). The tarsus bears several lobes, one of which bears a characteristic thumb that projects from its surface (A).

▣ = coxite; □ = telopodite; t = thumb, p = process, tp = tibial process.

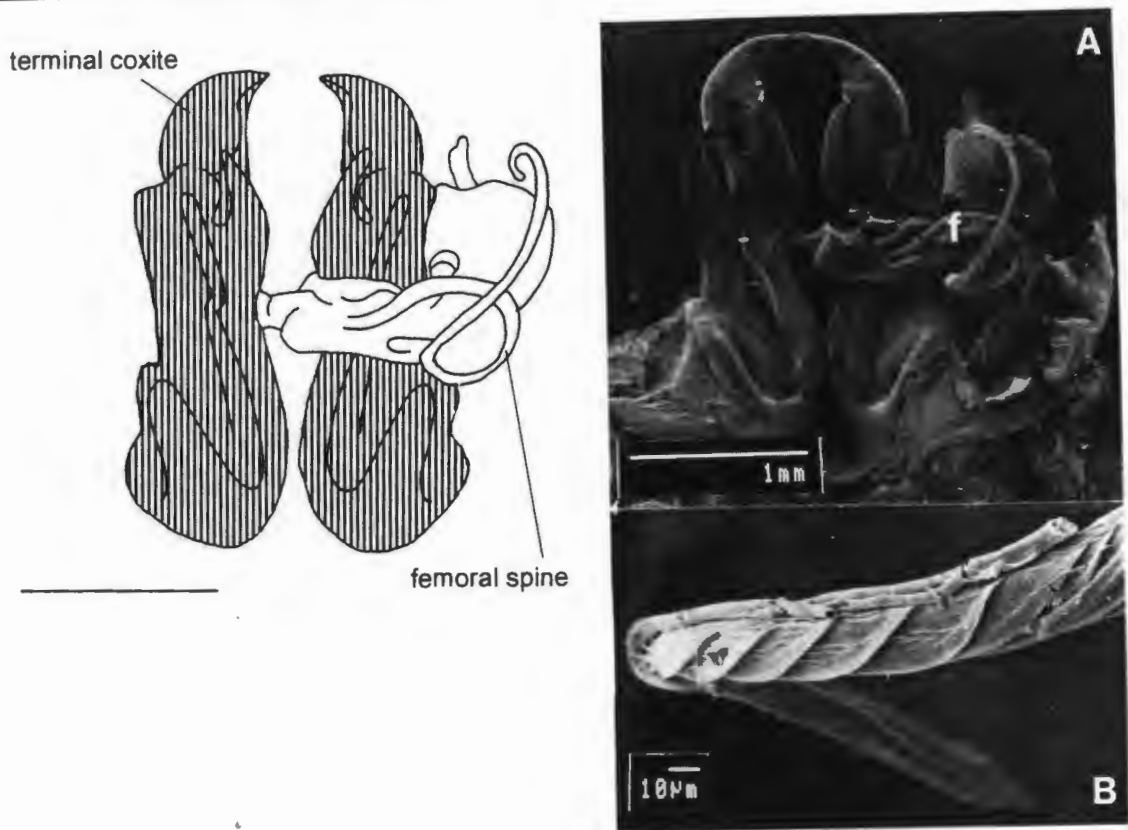


FIGURE 2.26 Oral view of the gonopods of *Chaleponcus hangklip* (Kraus).
Scale bar = 1mm.

PLATE 2.21 A. Oral view of *C. digitatus* gonopods (SEM).
B. Distal telopodite (SEM)

The gonopods of this species differ from the other *Chaleponcus spp* represented here in that the terminal lobe of the coxite tapers to a point (A). The telopodite bears a long tapering femoral spine with parallel ridges but no overlapping plates (B).

▨ = coxite; □ = telopodite; f = femoral spine.

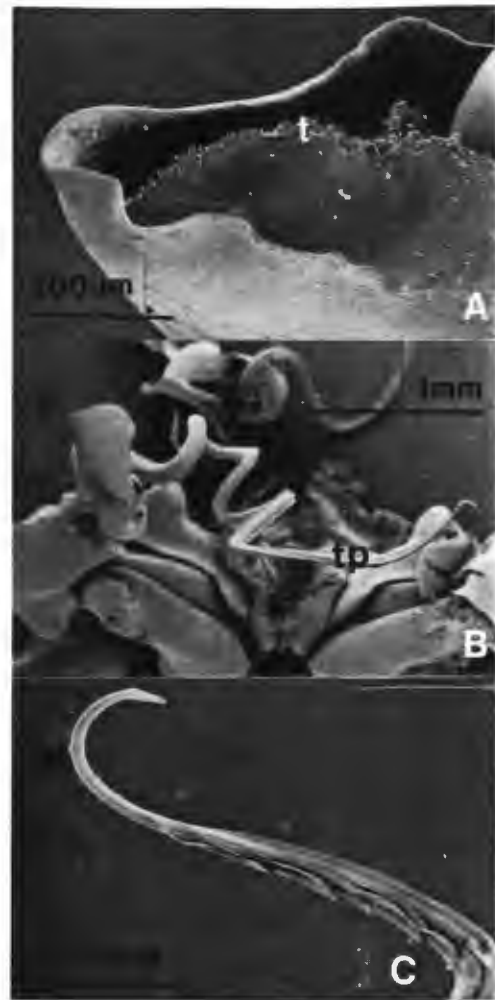
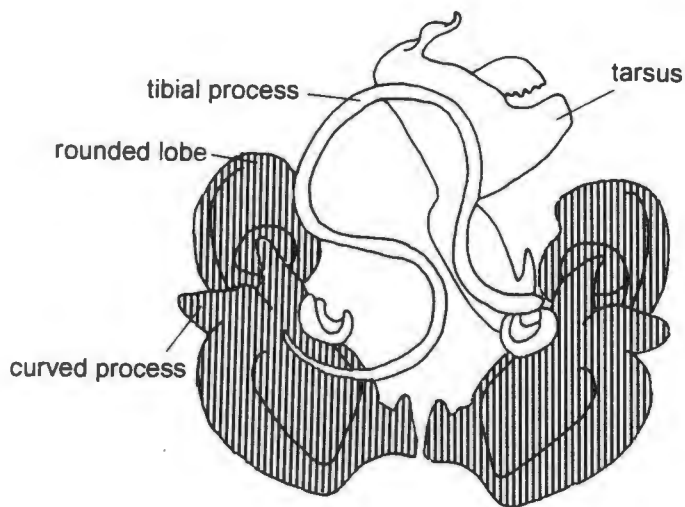


FIGURE 2.27 Aboral view of the gonopods of *Chaleponcus limbatus* (Attems).
Scale bar = 1mm.

- PLATE 2.22**
- A. Detail of distal oral coxite (SEM).
 - B. Gonopod with telopodite from above (SEM)
 - C. Detail of distal tibial process of telopodite (SEM)

The coxite of each gonopod of *C. limbatus* bears a curved lobate process and a rounded lateral lobe distally. At its terminal margin, the coxite bears rows of small teeth (A). The telopodite bears a pointed tibial process (B) with parallel overlapping plates (C) and a small tibial spine. The tarsus bears two lobes, the smaller of which bears a serrated process.

▣ = coxite; □ = telopodite; t = teeth, tp = tibial process.

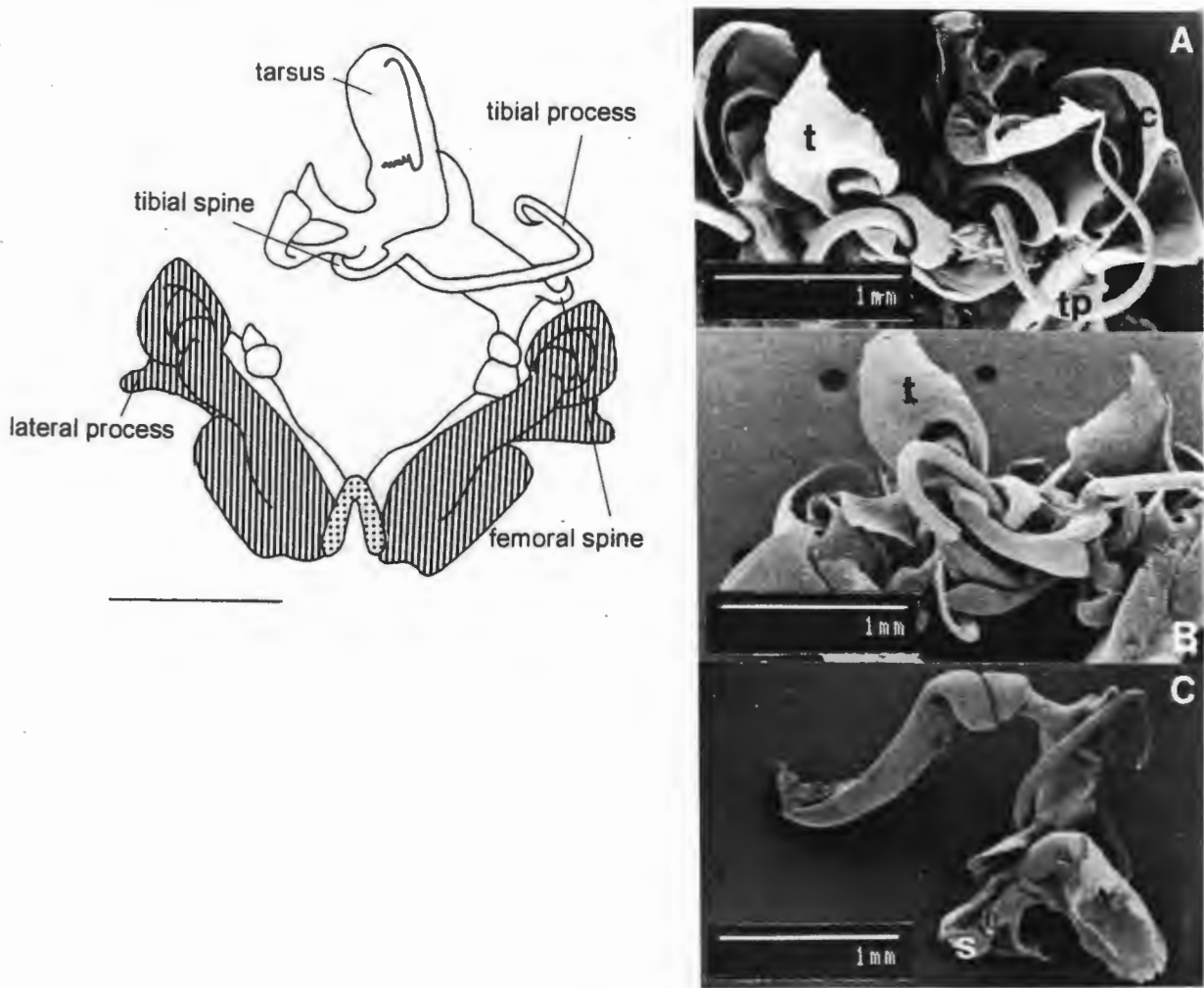


FIGURE 2.28 Oral view of the gonopods of *Chaleponcus oschei* (Kraus).
Scale bar = 1mm.

- PLATE 2.23**
- A. Aboral view of gonopods with coxite detail (SEM).
 - B. Aboral view of gonopods, telopodite detail (SEM)
 - c. Gonopod telopodite, removed from coxite gonocoel (SEM)

Each gonopod coxite is rounded distally (A) and bears a triangular lateral process that is clearly visible from the oral side (B). The telopodite twists about itself as it emerges from the coxite. It bears a pointed, finely furrowed tibial process that does not twist at its distal end, and several small tibial spines (A, c). The tarsus bears two leaf-shaped lobes, the smaller of which bears a spined pad at its terminal end (c).

▣ = coxite; □ = telopodite; c = coxite, t = tarsus, tp = tibial process, s = spined pad

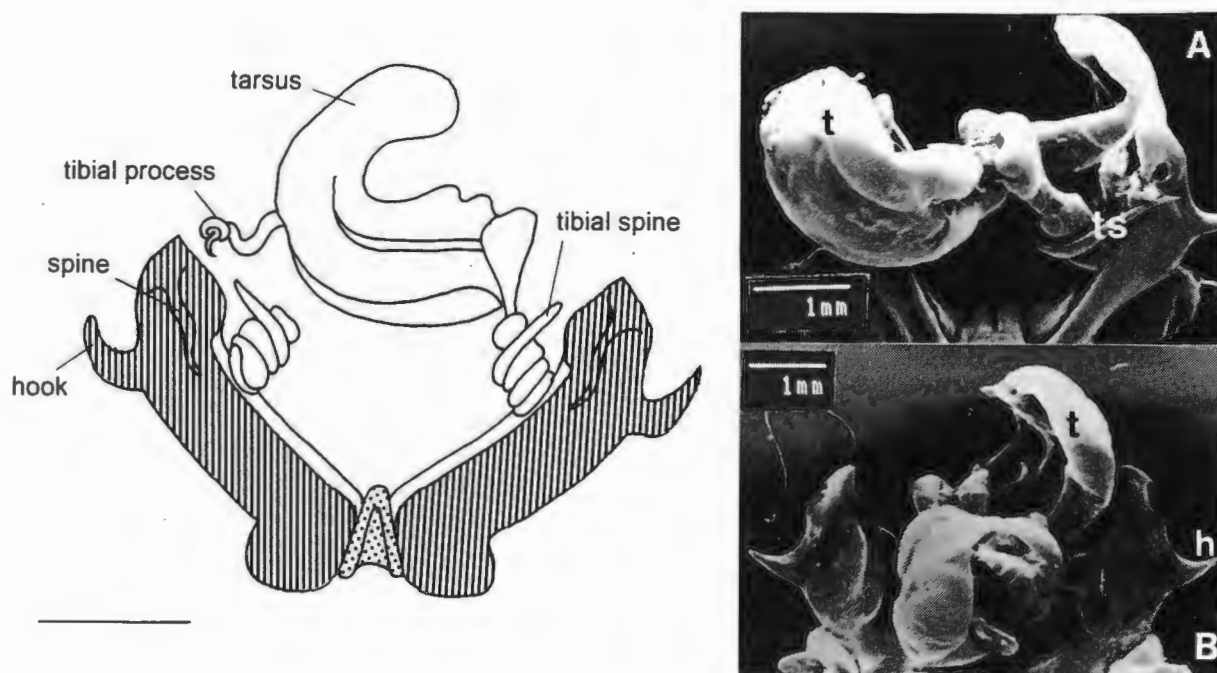


FIGURE 2.29 Aboral view of the gonopods of *Spinotarsus fiedleri complicans* (Kraus)
Scale bar = 1mm.

PLATE 2.24 A. Detail of left gonopod telopodite, oral view (SEM).
B. Distal gonopod coxites, oral view (SEM)

Each gonopod bears a large lateral hook and a medial downward projecting spine on the distal coxite (A, B). The telopodite bears a small tibial spine and a tapering tibial process that ends in a spiral (similar to that of *Spinotarsus sp. 1*), a large femoral spine and a large rounded tarsus (A, B).

▣ = coxite; □ = telopodite; t = tarsus, ts = tibial spine, h = hook

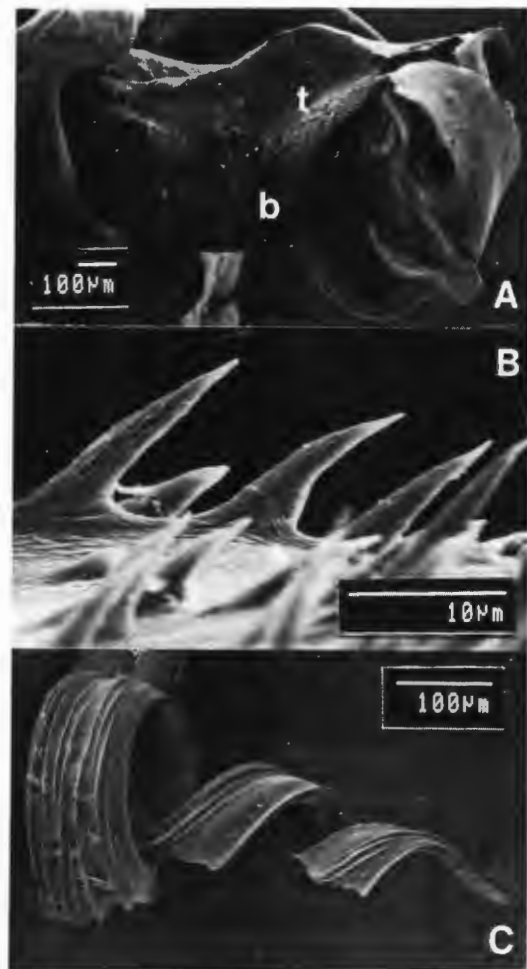
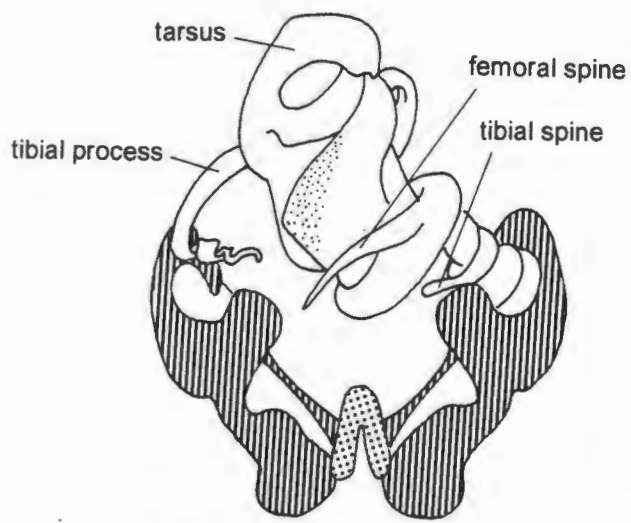


FIGURE 2.30 Oral view of the gonopods of *Spinotarsus skukuzicus* (Kraus)
Scale bar = 1mm.

- PLATE 2.25**
- A. Detail of tarsus of telopodite (SEM).
 - B. Detail of bristled region of telopodite tarsus (SEM)
 - c. Detail of the spiral tip of the tibial process (SEM)

Each gonopod of *S. skukuzicus* bears a smooth, rounded coxite. The telopodite bears a large curved tarsus (A) that bears a spine and a field of bristles (B). The long tibial process has parallel ridges and terminates in a spiral tip (C).

▨ = coxite; □ = telopodite; t = tarsus, b = bristles.

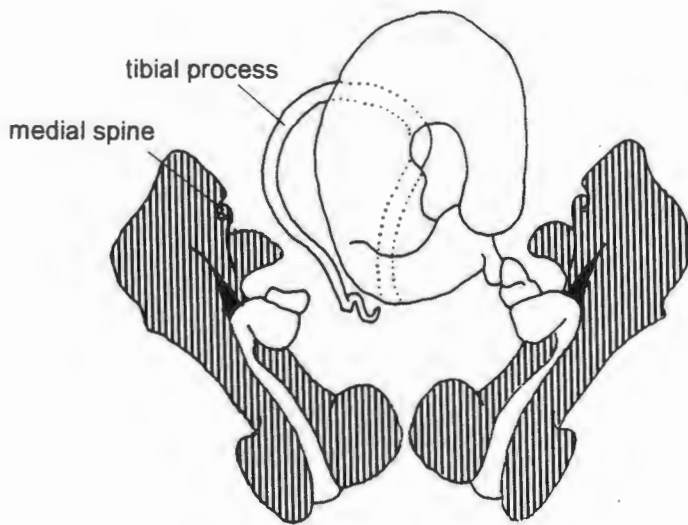


FIGURE 2.31 Oral view of the gonopods of *Spinotarsus* sp.1. Scale bar = 1mm.

- PLATE 2.26** A. Aboral view of left gonopod (SEM),
 B. Gonopod telopodite, removed from gonocoel of coxite (SEM),
 C. Distal tip of tibial process (SEM)

The gonopod coxite bears one lateral and one medial spine and a blunt medial process distally (A). The telopodite twists about itself before giving rise to a large scooped single lobed tarsus that bears a small serrated inner lobe (B). It bears a small femoral spine and a long pointed tibial process that is finely furrowed and curls at its distal end (C).

■ = coxite; □ = telopodite; s = medial spine, l = lateral spine, p = medial process, t = tarsus,
 tp = tibial process.

2.3.2 Functional morphology

The syncopulatory movements of the gonopods of five species of Spirostreptida were examined so as to provide a general overview of gonopod movement. Because the association of the telopodite and coxite does not differ within families, gonopod movements within families are predicted to be similar. In all species examined, ejaculates covered the distal coxites when copula pairs were separated.

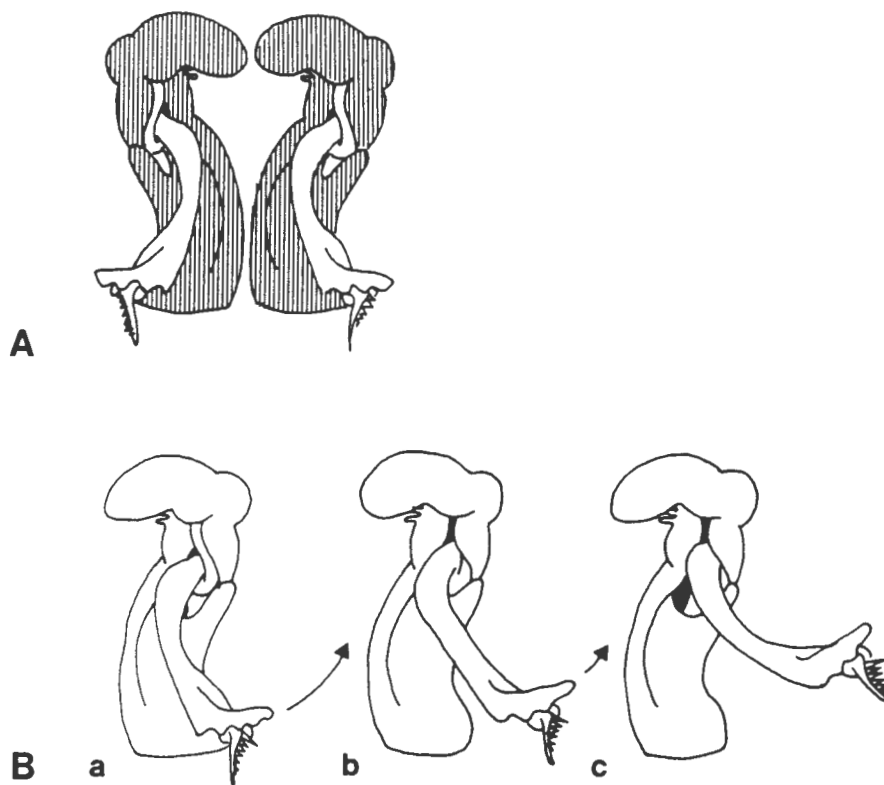


FIGURE 2.32 A. Diagrammatic representation of the gonopods of *Poratophilus diplodontus*, Aboral view.

Scale bar = 1mm. ▨ = coxite; □ = telopodite.

B. Functional morphology of the left gonopod of *Poratophilus diplodontus*, Aboral view, left gonopod. Arrows indicate future movement of the telopodite arm, which occurs as a result of its proximal retraction. See text for explanation.

Family HARPAGOPHORIDAE

The movement of the telopodite of *P. diplodontus* is presented in Fig. 2.32. During the first phase of copulation, the telopodite is retracted proximally, causing it to erect slightly and twist laterally about its axis (a - c). It is not retracted completely (as in the Spirostreptidae, see below) due to the femoral spine which rises vertically from the telopodite to beneath a distal fold of the coxite (Fig. 2.5a). Release of the telopodite results in it twisting back about its axis to its starting position (c - a). During its retraction-release cycle, *P. diplodontus* performs several partial retractions (a - b), retracts fully (a - c), performs several partial releases (c - b) and then retracts fully (c - a), from where the cycle begins again.

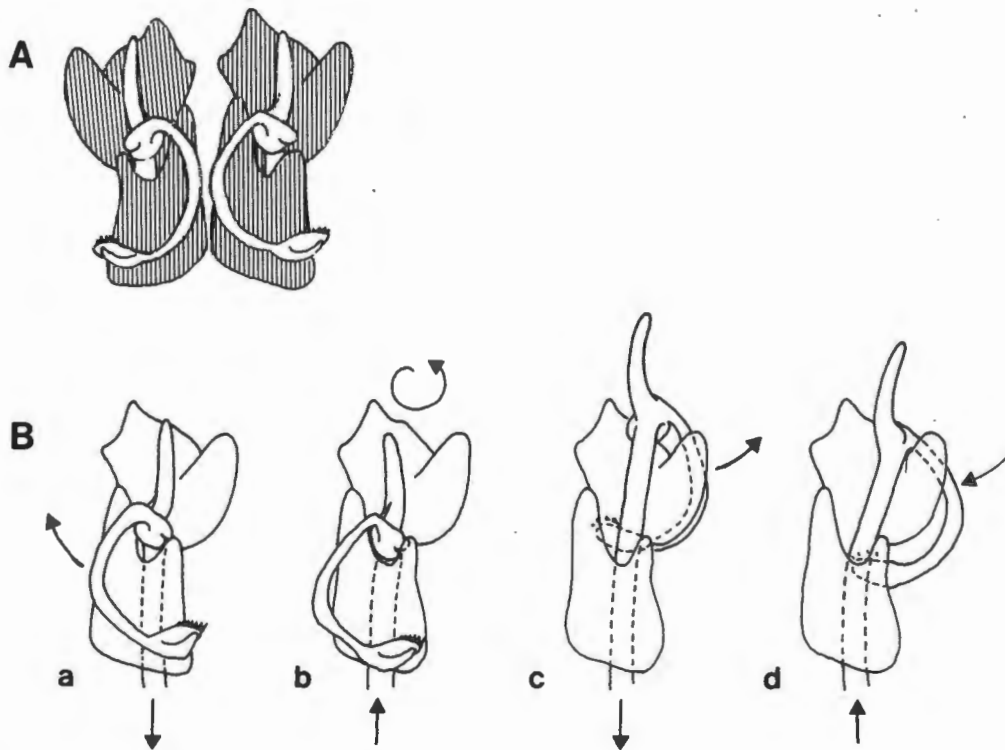


FIGURE 2.33 A. Diagrammatic representation of the gonopods of *Harpagophora polyodus*, Aboral view.

Scale bar = 1mm. ▨ = coxite; □ = telopodite.

B. Functional morphology of the left gonopod of *H. polyodus*. Aboral view, Arrows indicate future movement of telopodite, as effected by retraction at its base. See text for explanation.

Harpagophorid millipedes of the genus *Harpagophora* differ from *Poratophilus* species in that their femoral spines are not held beneath the distal coxites. Consequently the range of movements performed by *Harpagophora* telopodites is greater. In *Harpagophora polyodus* (Attems) males perform a series of small retractions (a - b), then release their telopodites completely such that their distal ends twist backwards over the coxite (c). Small retractions are performed from this position (c - d), followed by the retraction of the telopodite to its resting position (Fig 2.33).

Family SPIROSTREPTIDAE

The resultant movement of proximal retraction of the telopodite in *A. uncinatus* is described in Fig. 2.34. Initial retraction results in partial erection of the telopodite (a - b). The telopodite is then released, returning it to position a. This retraction - release motion between steps a and b is repeated about 20 times, and is then followed by full rapid retraction from step a through e, and release to a again. The entire sequence of telopodite partial and then full retraction is repeated throughout the duration of copulation.

During proximal telopodite retraction in *O. pyrhocephalus* (Fig. 2.35), the convoluted telopodite arm traverses the bridge formed by the margin at the fold of the coxite, and causes the scoop at the distal end of the telopodite to twist within the vulval sacs. The distal end rises (b); twists forward (c); twists back (d); twists forward (e) and then flips round to make contact with the spined region of the coxite. Release of the telopodite results in the scoop brushing downwards against the spines. It then resumes its original position within the vulvae. Multiple retraction-release cycles are performed by males during copulation (*O. pyrhocephalus* functional morphology after Barnett 1991).

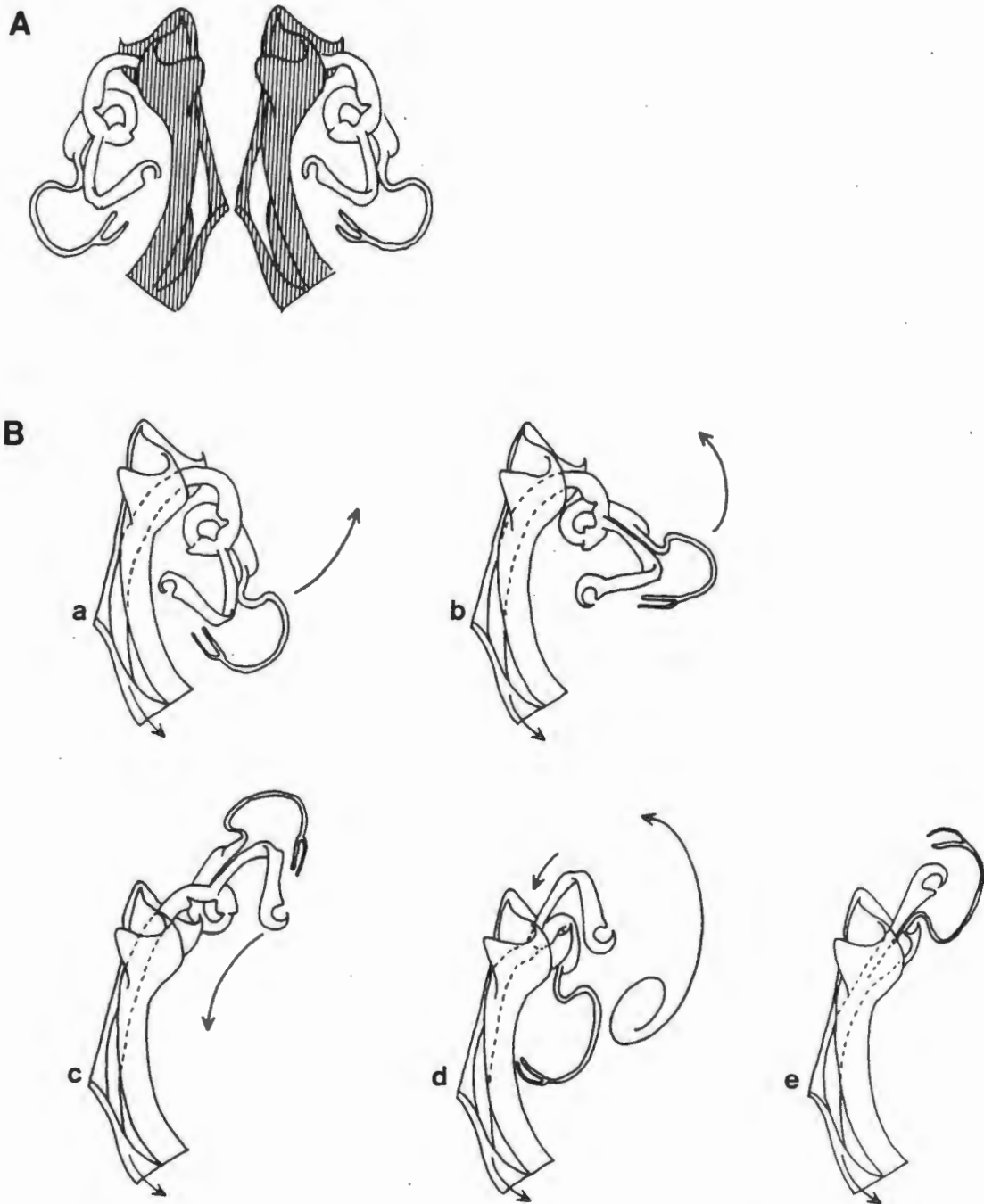


FIGURE 2.34A. Diagrammatic representation of the gonopods of *Alloporus uncinatus*, Oral view. Scale bar = 1mm.

▨ = coxite; □ = telopodite.

B. Functional morphology of the right gonopod of *Alloporus uncinatus*. Oral view represented. Arrows indicate future movement of the telopodite arm, which occurs as a result of its proximal retraction. See text for explanation.

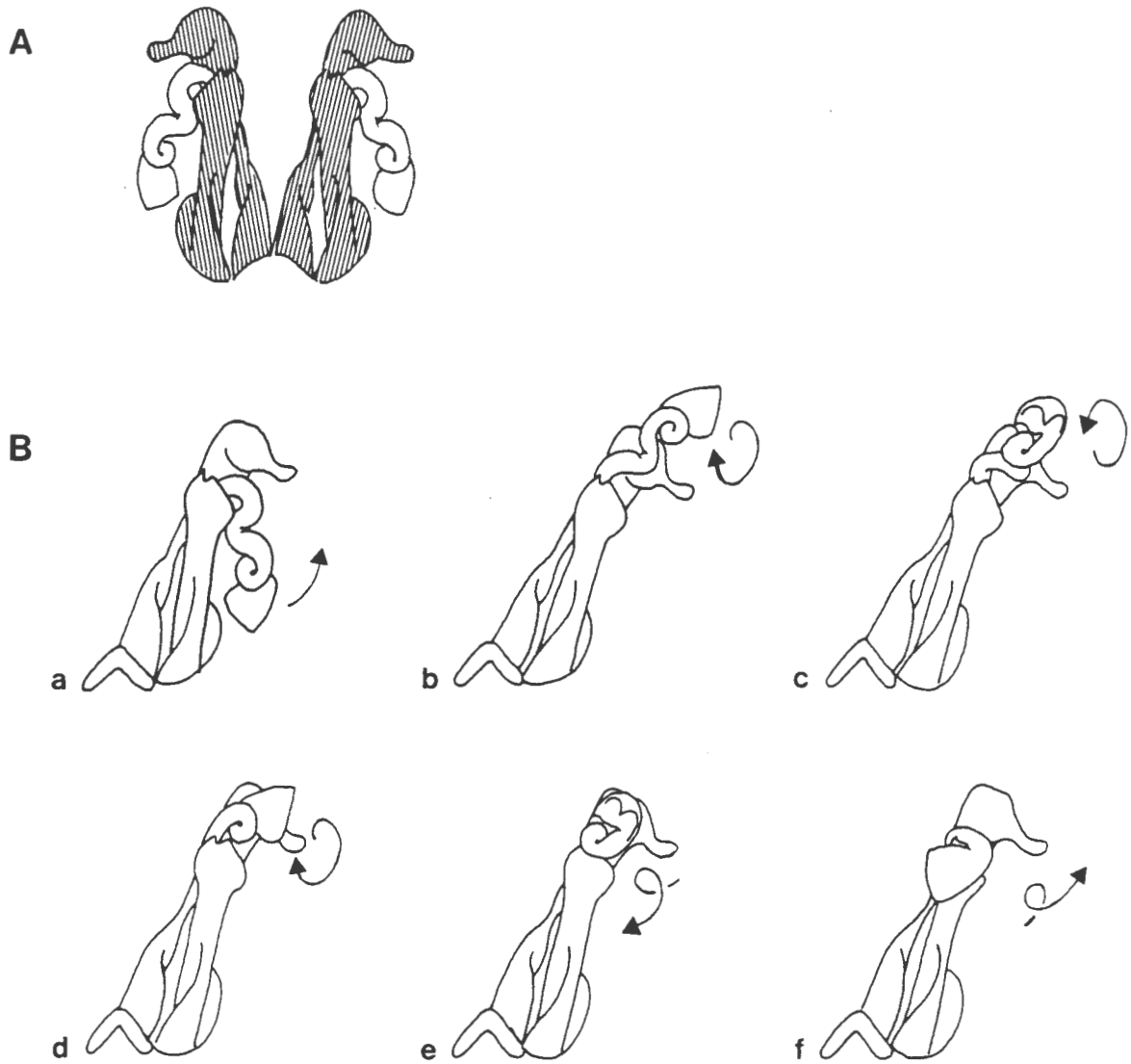


FIGURE 2.35 A. *Orthoporoides pyrhocephalus* gonopods, oral view. Scale bar = 1mm. ▨ = coxite; □ = telopodite. B. Functional morphology of the right gonopod of *O. pyrhocephalus* Oral view represented. Arrows indicate future movement of the telopodite arm, which occurs as a result of its proximal retraction See text for explanation (after Bamett 1991).

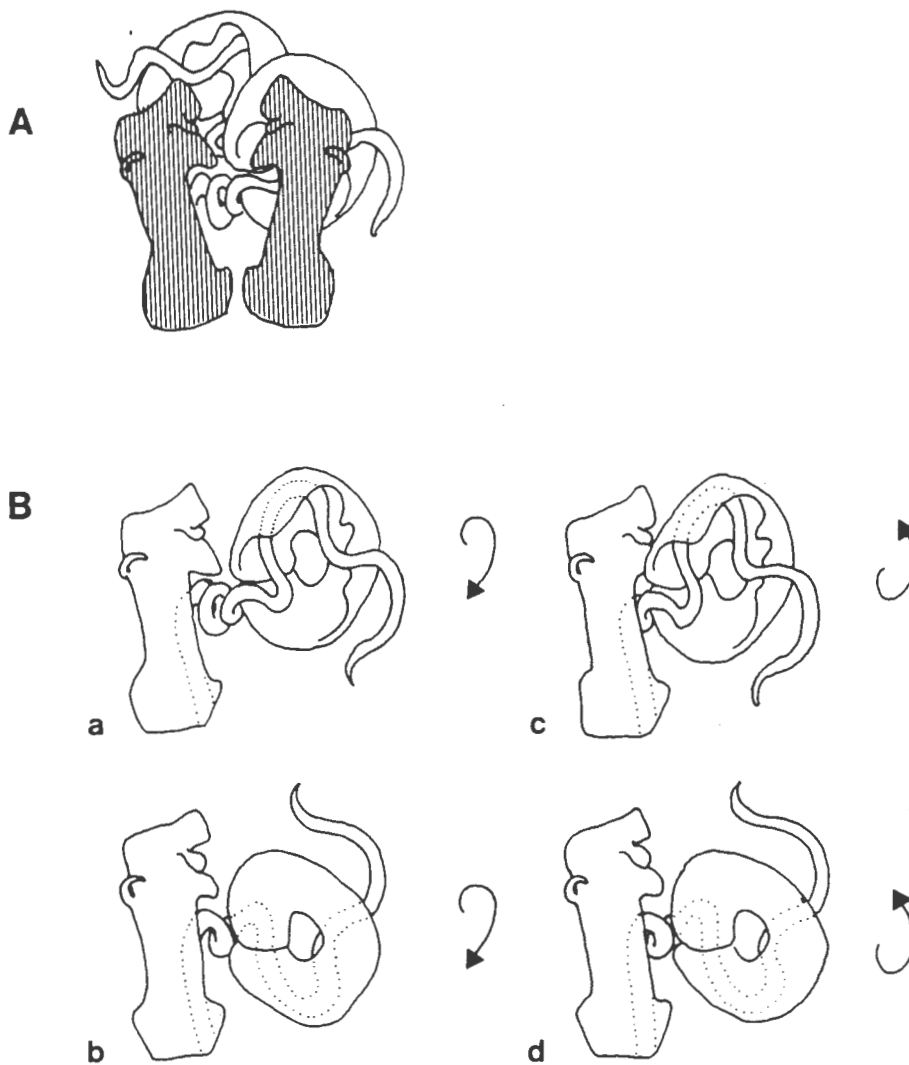


FIGURE 2.36 A. Diagrammatic representation of *Spinotarsus sp.1* gonopods. oral view. Scale bar = 1mm.

▨ = coxite; □ = telopodite.

B. Functional morphology of the left gonopod of *Spinotarsus sp.1*, oral view. Arrows indicate future movement of the telopodite arm, which occurs as a result of its proximal retraction. See text for explanation.

Family ODONTOPYGIDAE

The telopodite retraction-release cycle of *Spinotarsus sp.1* is represented in Fig. 2.36. Retraction of the arm results in tension across the telopodite's coils and causes the distal arm to twist about its own axis. This results in whip-like movements of the tibia and causes the tarsal scoop to flip forwards (b); forwards again (c); and then backwards (d) and backwards again (a).

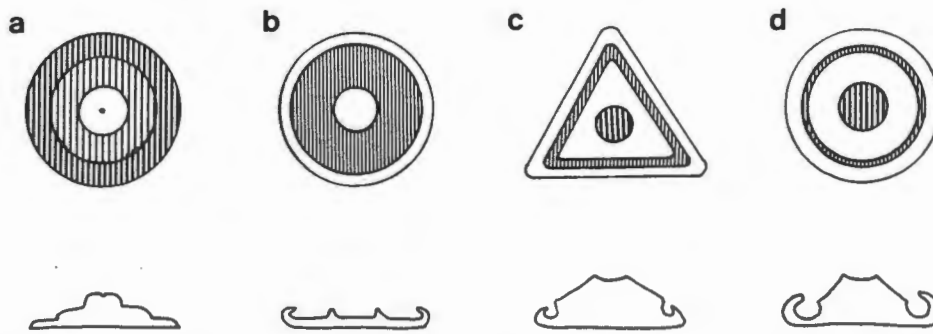


FIGURE 2.37 Diagrammatic representation of Spirostreptida sperm morphology, showing oral (top row) and lateral (bottom row) views. **a.** Harpagophoridae sperm morphology. **b.** Odontopygidae sperm morphology. **c.** Spirostreptidae sperm morphology, triangular morph (*Alloporus* species). **d.** Spirostreptidae sperm morphology, circular morph (*Triaenostreptus* and *Orthoporoides* spp.)

2.3.3 Sperm morphology

Ejaculates were densely packed with spermatozoa when removed from the gonopod coxites. All sperm were aflagellate, conforming to previous descriptions, and therefore probably non-motile. There was no great variability in general sperm size, and no marked relationship between general body size and sperm size. However, sperm are species-specific, and show intrafamily similarities and interfamilial differences that provide strong supporting evidence for current classification of the Spirostreptida.

Within families, sperm correspond to a characteristic form (but see the Spirostreptidae) (Fig. 2.37). All have centrally placed acrosome complexes and peripheral grooves that divide the sperm base into two. The peripheral grooves form ridges that extend outwards towards the margins in patterns that seem to be species-specific. Both spermatophores (two sperm conjoined) and unilateral sperm were found in the ejaculates of all species examined. This is a condition that persists in the female (see Chapter 3).

Harpagophoridae spermatozoa (Fig. 2.37a; Plate 2.27 A-H) are disc shaped and range between 8.5 μm and 11.6 μm in diameter. Acrosomes are raised with narrow central depressions that resemble central dots when viewed with light microscopy, and form shallow grooves where they meet the sperm base. A second shallow peripheral groove forms a marginal rim that divides the base into upper and lower levels. Edges of the lower base do not recurve and taper sharply.

Odontopygidae spermatozoa (Fig. 2.37b; Plate 2.27 P-R) are also disc-shaped and range from 8.2 μm to 13.3 μm in diameter. Acrosomes are central, relatively wider than the harpagophoridae and are raised at their edges but centrally depressed. One or two peripheral grooves are always present, the outer groove being formed by the edges of the base recurving over it.

The Spirostreptidae differ from the Harpagophoridae and Odontopygidae in that two sperm morphs occur (Fig. 2.37c, d; Plate 2.27 I-O) and some ejaculates were surrounded by a thick coarsely granular fluid. Species from the genus *Alloporus* produce triangular sperm (9.6 μm - 11.7 μm). Acrosomes are centrally placed with depressed centres and gently sloping sides that form a shallow groove where they meet the base. Deep peripheral grooves divide the base into upper and lower levels. They are formed by the recurved edges of the lower base. Sperm from other Spirostreptidae genera are disc shaped (8.2 μm - 10.7 μm) and recurve slightly over the base to form the peripheral groove that divides the base into two levels. Acrosomes are well developed and are raised with central depressions and gently sloping sides.

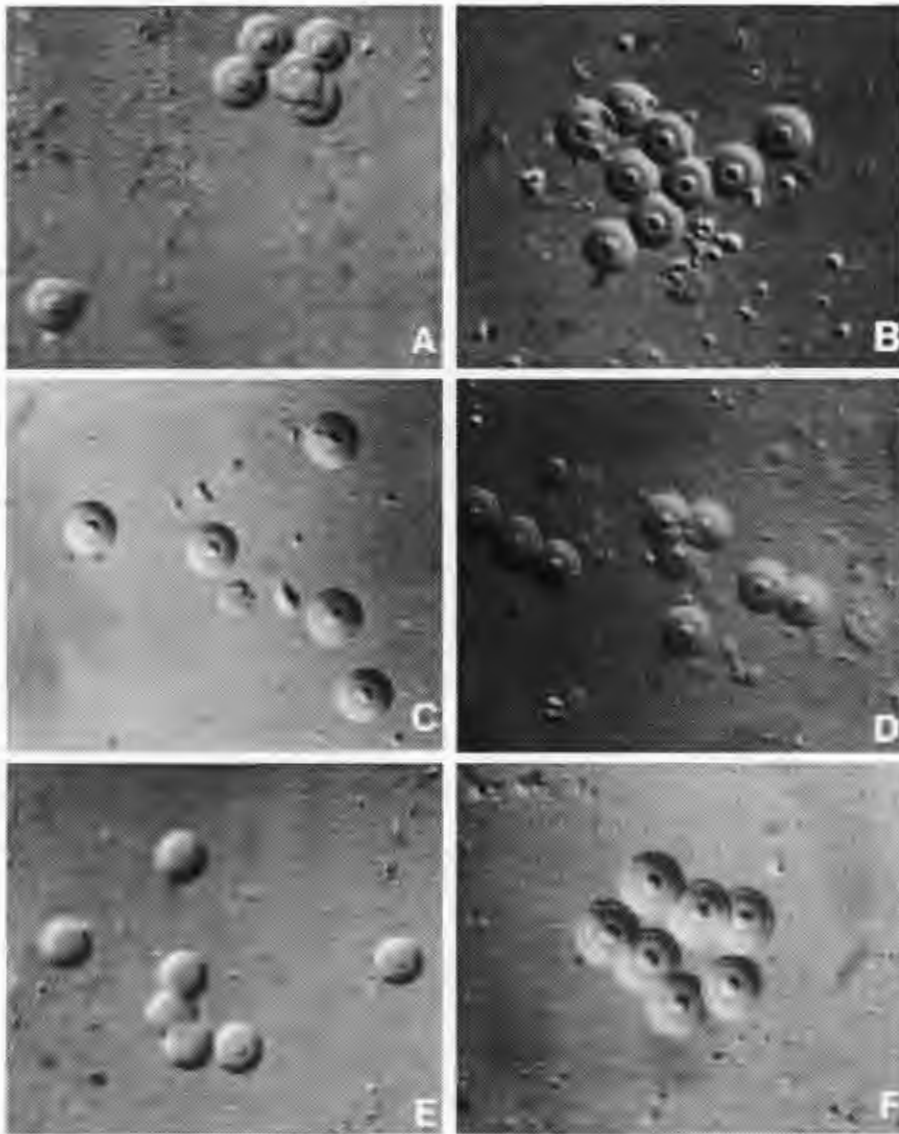


PLATE 2.27 Spirostreptida sperm (Mean sperm diameter is given in brackets).

Harpagophoridae: A. *Harpagophora levis* (11.05 μ m), B. *Poratophilus diplodontus* (10.2 μ m), C. *Poratophilus similis* (11.33 μ m), D. *Poratophilus* sp.1 (10.76 μ m), E. *Poratophilus* sp.2 (10.2 μ m), F. *Poratophilus* sp.4 (11.62 μ m), (...continued overleaf)

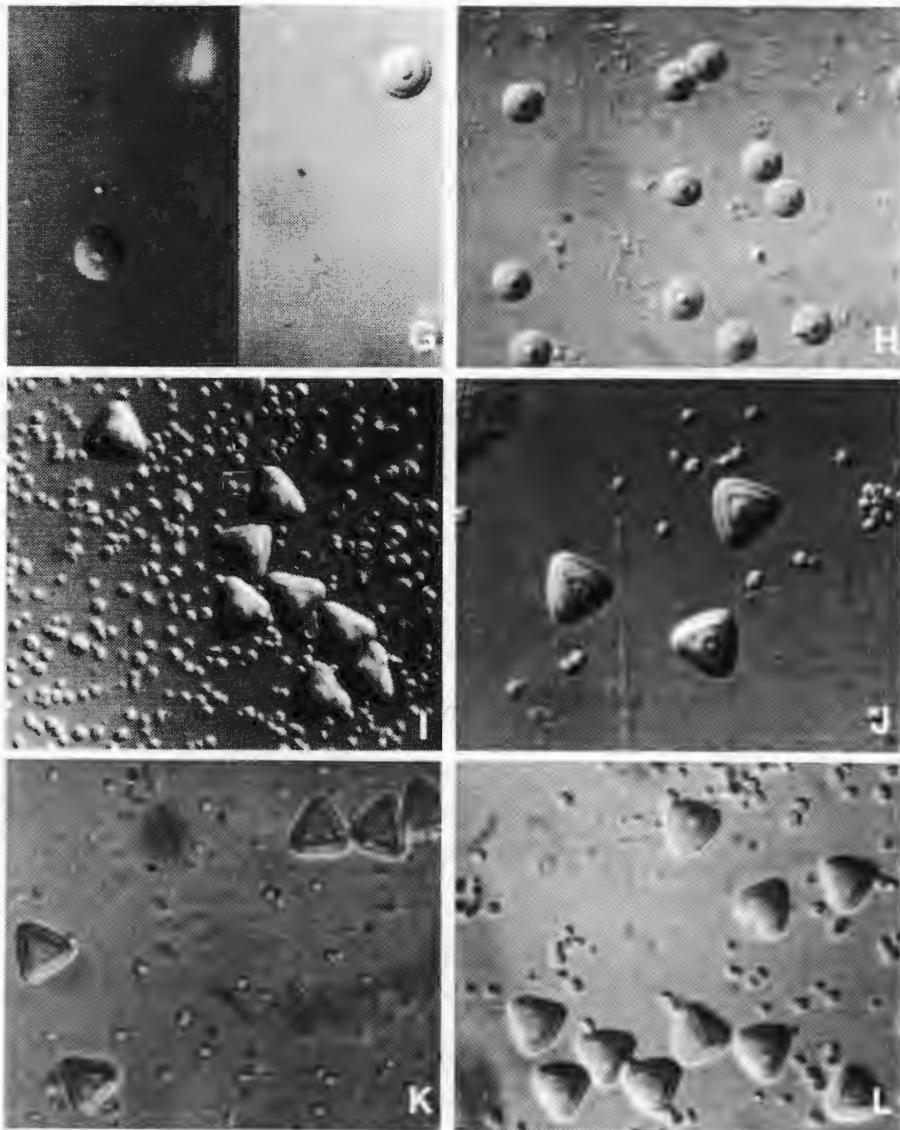


PLATE 2.27 (CNTD). G. *Poratophilus* sp.5 (10.31 μ m), H. *Poratophilus* sp.6 (8.5 μ m), Spirostreptidae: I. *Alloporus flavilis* subsp.(11.78 μ m), J. *Alloporus rugifrons* (10.31 μ m), K. *Alloporus uncinatus* (10.2 μ m), L. *Alloporus* sp.1 (9.63 μ m), (...continued overleaf)

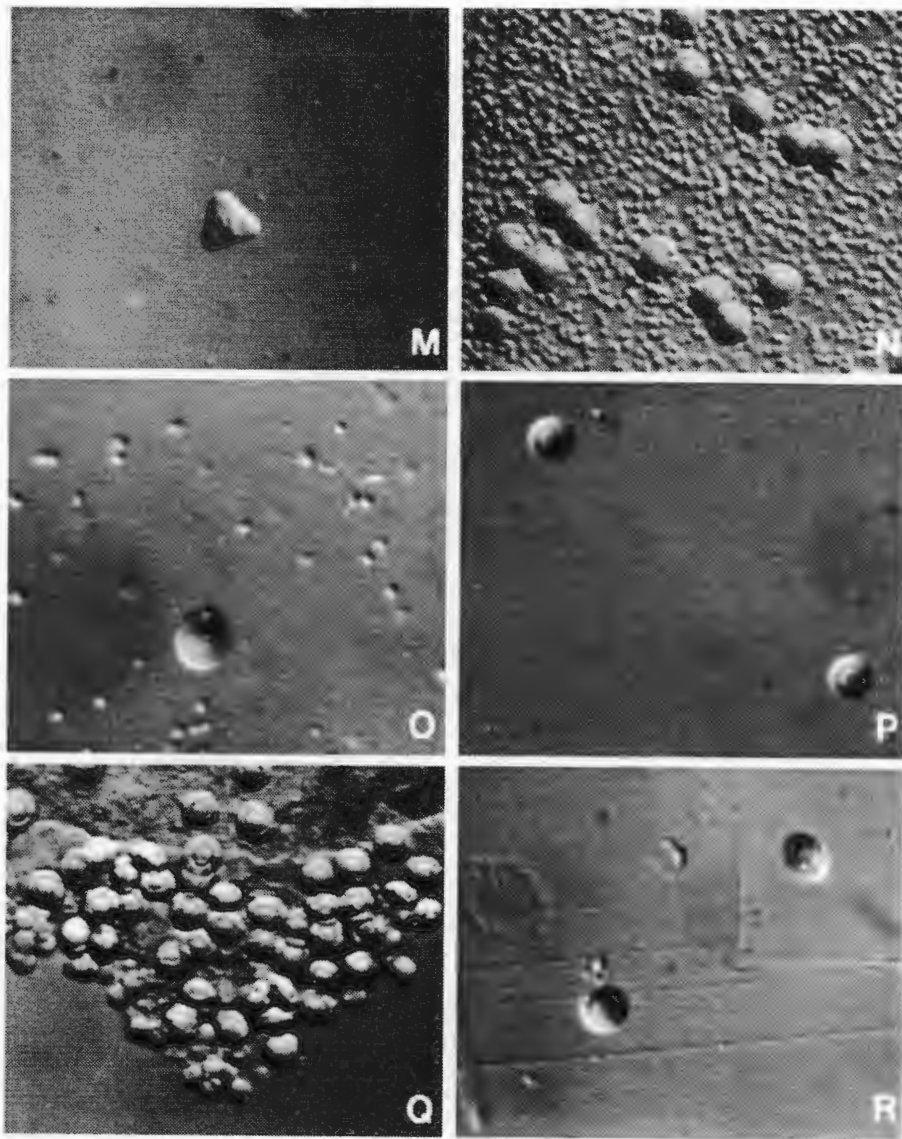


PLATE 2.27 (CNTD). M. *Alloporus* sp.2 (10.77 μ m), N. *Triaenostreptus* sp. (9.8 μ m), O. *Orthoporoides pyrhocephalus* (9.92 μ m), Odontopygidae: P. *Chaleponcus limbatus* (8.5 μ m), Q. *Spinotarsus* sp.2 (13.3 μ m), R. *Spinotarsus* sp.3 (13.3 μ m).).

2.4 Discussion

The association of gonopod components is similar within families and more particularly within genera, and it is predicted that the functional morphology and mechanisms of competition are conserved within these groups. With the exception of the Spirostreptidae coxites, Spirostreptida telopodites are the most complex regions of the gonopods. Several hypotheses are considered as possible explanations for the evolution of gonopod and particularly telopodite complexity. Although Eberhard (1985) rejects all hypotheses except that of female choice at the general level, he does concede that some may be relevant in explaining aspects of genital morphology in certain groups. Further, in some taxa, genitalia are considered to be adaptive in several ways (in addition to their basic role in sperm transfer). For example, in both the beetle *Tenebrio molitor* (Gage 1992) and the bushcricket *Metaplastes ornatus* (von Helversen and von Helversen 1991) genitalia are believed to function in both internal courtship and displacement.

2.4.1 Gonopods in sperm competition

The functional morphology of all five species examined revealed cyclic sequences of movement that may facilitate ejaculate transfer, redistribution and/or mixing.

a. Gonopods in sperm transfer

Sperm immotility has implications for processes of sperm transfer. Where sperm are motile, they can independently change their position within the females' storage structures (Sivinski 1984), and placement may be less critical. Where sperm are non-motile, if stochastic events are excluded, their positioning within the vulvae is dependent on placement by the male genitalia and subsequent female processes.

The role of the telopodite in processes of sperm transfer is not clear. According to Demange (1959), sperm are translocated from the penes to the base of the coxites, from where they are pumped up the telopodite canal and into the vulvae. However, the observation that the distal coxites were covered with ejaculate components when copula pairs were separated suggests that this may not be the case. An alternative route for sperm transfer may be directly from the penes to the distal coxites. Importantly, for the telopodites to be implicated in ejaculate transfer from the distal coxites, they need

to make contact with them during gonopod movement cycles (at the onset of copulation sperm are transferred from the penes to the distal coxite).

For example, in *A. uncinatus* and *O. pyrhocephalus*, males perform a series of partial retractions before fully retracting the telopodite arm and bringing it in contact with the coxite. Sperm may be translocated from the coxite to the distal telopodite with complete retraction (Figs 2.34, 2.35). Evidence for the translocation of ejaculates to different parts of the gonopods is found in the Chordeumatida where sperm are moved from sperm vesicles on the syncoxite to small brushes that move over the spermathecal openings (Tadler 1993) and in the Parajulidae in which sperm pass from the anterior to the posterior gonopods (Matthews and Bultman 1993).

In both the Harpagophoridae and Odontopygidae, distal telopodites do not touch the coxite at any point of the movement cycle and it is unlikely that selection for efficient transfer accounts for telopodite complexity in these families.

b. Gonopods in sperm repositioning or mixing

The functional morphology of all five species examined revealed complex cyclic sequences of movement that could potentially function to redistribute or mix ejaculates within the vulvae. Redistribution of (non-motile) sperm closer to the point of ova emergence could improve a male's fertilisation advantage (Walker 1980), and mixing of the contents of the vulval sacs could nullify position advantages that may otherwise have been accrued due to ejaculate stratification.

c. Gonopods in sperm removal

In order to actuate direct sperm removal, gonopods would need to bear morphological devices with which to manipulate rival sperm. In species shown to displace sperm (Odonata: Waage 1982, 1984, 1986a, 1986b; Miller 1982, 1990; McVey and Smittle 1984; Siva-Jothy 1984, 1987a; Michiels and Dhont 1988; Orthoptera: von Helverson and von Helverson 1991; Coleoptera: Gage 1992) the morphological devices that have evolved to facilitate displacement include scoops, spines, spurs and flagellae with overlapping barbs.

Scoop-like structures occur on the telopodites of several species of millipede belonging to the families Odontopygidae and Spirostreptidae. These vary in form and in their position on the telopodite. In *O. pyrhocephalus*, the telopodite terminates in a spade-like structure. In *Spinotarsus* spp., the tarsus is often scoop-shaped. In *Alloporus* spp., a trowel-like scoop often occurs half way up the telopodite canal-bearing arm where it bifurcates. The tibial arms of Odontopygidae telopodites frequently bear ridges or backwardly overlapping plates at their distal ends. Harpagophoridae telopodites terminate with a complex association of lobes and rigid comb-like structures. Spines are found on the distal oral coxites of the Spirostreptidae. These spines vary in form from stout pitted spines to long hair-like spines that are pitted in some species and not in others.

Having described the potential displacing devices, it must be noted that in order to directly effect rival sperm displacement, genitalia must have access to the regions where sperm are stored. This is discussed in Chapter 3, in which the importance of understanding the incopula interaction of male and female genitalia before ascribing function to structure, is emphasised. The possibility that gonopods function in indirect sperm displacement via the induction of females to manipulate rival sperm is discussed below.

2.4.2 Gonopods in female choice

a. Gonopods as cues to fitness

Genitalia may function as cues to male fitness (Eberhard 1985). As in several other taxa (Eberhard 1996 p76), millipede genitalia are generally poor indicators of body size (Barnett 1991; Barnett *et al.* 1991, Dangerfield *et al.* in prep.; there are exceptions see Eberhard 1985 p.79). However, this does not mitigate against their use as cues to male quality: selection can be based on arbitrary traits where these do not conflict with natural selection. For example, in the beetle *Chelymorpha alternans*, paternity and genital size are positively correlated, even though genitalia do not scale with other aspects of male morphology (Rodriguez 1994a cited Eberhard 1996). Where selection is based on arbitrary traits, runaway selection may account for genital complexity (Fisher 1938).

b. Gonopods in internal courtship

Genitalia can also function to stimulate females to preferentially accept the ejaculate of a particular male (Eberhard 1996). Although sensory receptors in the vulvae have not been found (Tadler 1996), stimulatory functions have been attributed to the gonopods of the Chordeumatida (Tadler 1993) and the Julida (Tadler 1996, Haaker and Fuchs 1970). The nature and function of the stimulation is generally unclear. It has been suggested that the Julida stimulate females to eject the sperm of previous mates (Tadler 1996 citing Blower 1985), and that the Spirostreptida use their distal telopodites to stimulate egg release (Demange 1959).

In the Chordeumatida stimulation is associated with a contiguous association of gonopods and vulvae throughout copulation (*Craspedosoma* spp., Tadler 1993). In the Spirostreptida and Julida this is not possible due to augmented gonopod mobility. In the Julida, the stimulatory cues are in motion rather than stationary and take the form of a series of rhythmic intromissions prior to sperm transfer (Haaker and Fuchs 1970, Eberhard 1985). The gonopod telopodites of the Spirostreptida perform retraction-release cycles during copulation, and these movements may be analogous to the rhythmic intromissions of the Julida. Rhythmic genitalic movements are a widespread phenomena that are poorly understood due to difficulties in distinguishing between stimulatory functions and sperm manipulation activities (Eberhard 1994; 1996). Only with an understanding of female morphology and the mechanisms of sperm transfer and displacement can the potential role of the genitalia in stimulation be appreciated.

2.5 Summary

Gonopod complexity in the Spirostreptida cannot be accounted for by sperm transfer alone. Structural evidence implicates sexual selection via both cryptic female choice and sperm competition. All hypotheses are contingent upon female genital morphology and the interaction of male and female genital components, and these are considered in Chapter 3.

CHAPTER THREE

VULVAL MORPHOLOGY: FEMALE CONTROL OF PATERNITY?

3.1 Introduction

The vulvae constitute the syn- and postcopulatory arena for competitive interactions and may afford females control over sperm distribution patterns and ultimately paternity (Walker 1980; Eberhard 1985). Processes of sperm movement and subsequent distribution are integral to the mechanics of sperm competition. Because these are dependent on the functional morphology of the vulvae, cognisance of vulval morphology is essential for the integration of female-mediated processes into an understanding of the mechanisms of competition.

3.1.1 Millipede vulval morphology

Vulvae are taxonomic characters in some Diplopoda families and only in these is their external (e.g. Brölemann 1935; Verhoeff 1936; Kurnik and Thaler 1985; Kurnik 1988) and internal (Tadler 1993; 1996) morphology well documented. Spirostreptida vulval morphology is less well understood. Seminal work from the turn of the century (Fabre 1855; Efferberger 1909; Reinecke 1910; all cited Brölemann and Lichtenstein 1919; Brölemann 1917; Brölemann and Lichtenstein 1919) has tended to be overlooked or perhaps ignored by subsequent investigations (e.g. Kraus 1968). One consequence of this is a plethora of synonyms (in the taxonomic investigations of the vulvae, several terms often describe the same structure), leading to vague and often contradictory explanations of vulval functional morphology (e.g. Blower 1985; Hopkin and Read 1992 but see Demange 1959).

In this chapter, external vulval morphology is described for 20 Spirostreptida species (8 Spirostreptidae spp; 6 Harpagophoridae spp; 6 Odontopygidae spp). A more detailed account is provided for three of these: *Alloporus uncinatus* (Spirostreptidae), *Poratophilus diplodontus* (Harpagophoridae) and *Spinotarsus fiedleri complicans* (Odontopygidae).

3.2 Methods

Spermathecal musculature, structure and ultrastructure were examined using light microscopy (LM) and scanning electron microscopy (SEM).

Following mating and a predetermined time delay, females were sacrificed in ethyl acetate killing jars and their vulvae were removed. Vulvae were either fixed and stored in 70% ethanol for histological processing, or examined immediately by placing them on glass slides and photographing them with a Wild M400 light microscope with camera back. Schematic reconstructions were produced from these photomicrographs with the aid of a Eiki 840 AXE slide projector with built in screen. The opaque nature of the vulval sacs made it relatively easy to distinguish the size and form of the bursae through the vulval membranes. It is noted that vulval membranes are distendable and as in other taxa (Miller 1982; Siva-Jothy 1985) their morphology may be altered by male appendages during copulation.

3.2.1 Histology

Specimens that had been stored in 70% ETOH were embedded in paraffin wax (melting point 56 - 60°C). Sections of approximately 3 µm thick were cut on a rocking microtome, and stained with either Mallory's triple connective stain or hematoxylin and eosin (Humanson 1962). (Mallory's stain allows tissue to be distinguished on the basis of colour differences. Hematoxylin and eosin give good cell structure definition). Sections were mounted on glass slides in DPX (Gurr) and examined with bright field. Photomicrographs were taken with a Leica Lab K microscope with camera back.

3.2.2 Scanning Electron microscopy (SEM)

Vulvae were removed, dehydrated and prepared for SEM as in Chapter 2. They were viewed and photographed with a Leica 440 scanning electron microscope.

3.2.3 Association of male and female genitalia

Matings were generated in glass arenas (30 cm x 50 cm x 20 cm) containing equal numbers of males and females (each sex, $n = 10$). Syncopula pairs were removed from the arena, frozen in liquid nitrogen and freeze dried. Copula pairs were dissected as they thawed to reveal the position of the gonopods within the female reproductive tract, and dissections were photographed.

3.3 Results

3.3.1 General vulval morphology

Although they have been (incorrectly) described as undifferentiated sac-like structures (Kraus 1968; Barnett *et al.* 1995), Spirostreptida vulvae are similar to those of other Diplopoda and correspond to the general description of Brölemann and Lichtenstein (1919). They are formed from an invagination of the body wall and open behind the second pair of legs. Each vulva consists of a membranous sac with an internal structure, the **bursa**, that is formed by a protrusion of its membranes. Bursae can be located deeply within the **vulval sacs** (Odontopygidae and Spirostreptidae) or close to the surface, protruding from the gonopore (Harpagophoridae) (Plates 3.1, 3.2, 3.3). The position of the bursae within the vulval sacs is conserved within families (Figs 3.1, 3.2, 3.3).

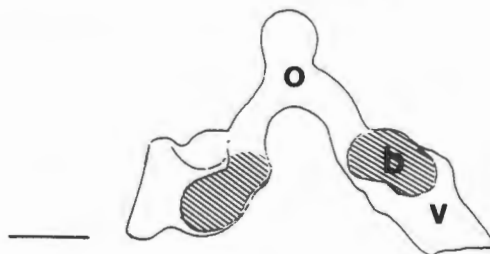
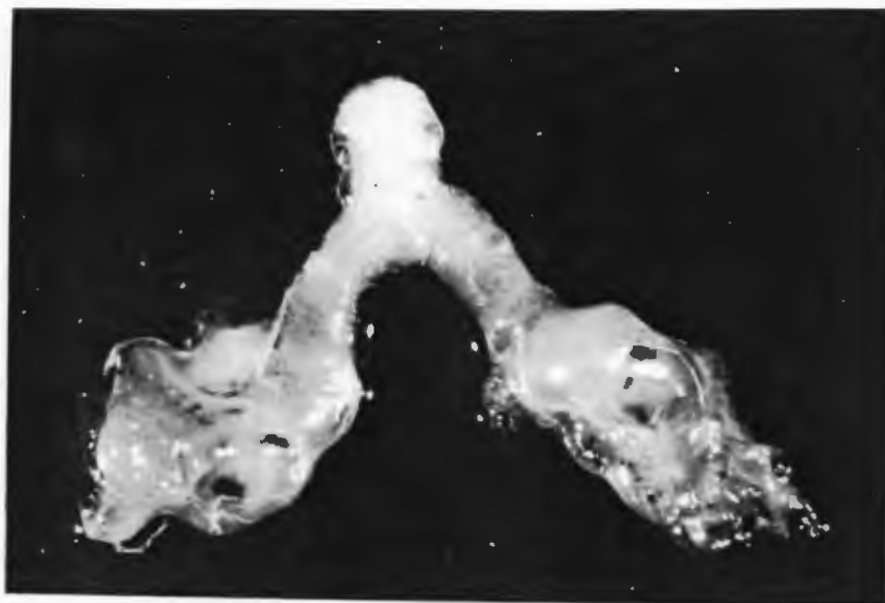


PLATE 3.1 Vulvae of *A. uncinatus*. b = bursa; o = oviduct; v = vulval sac (x14). Scale bar = 1mm.

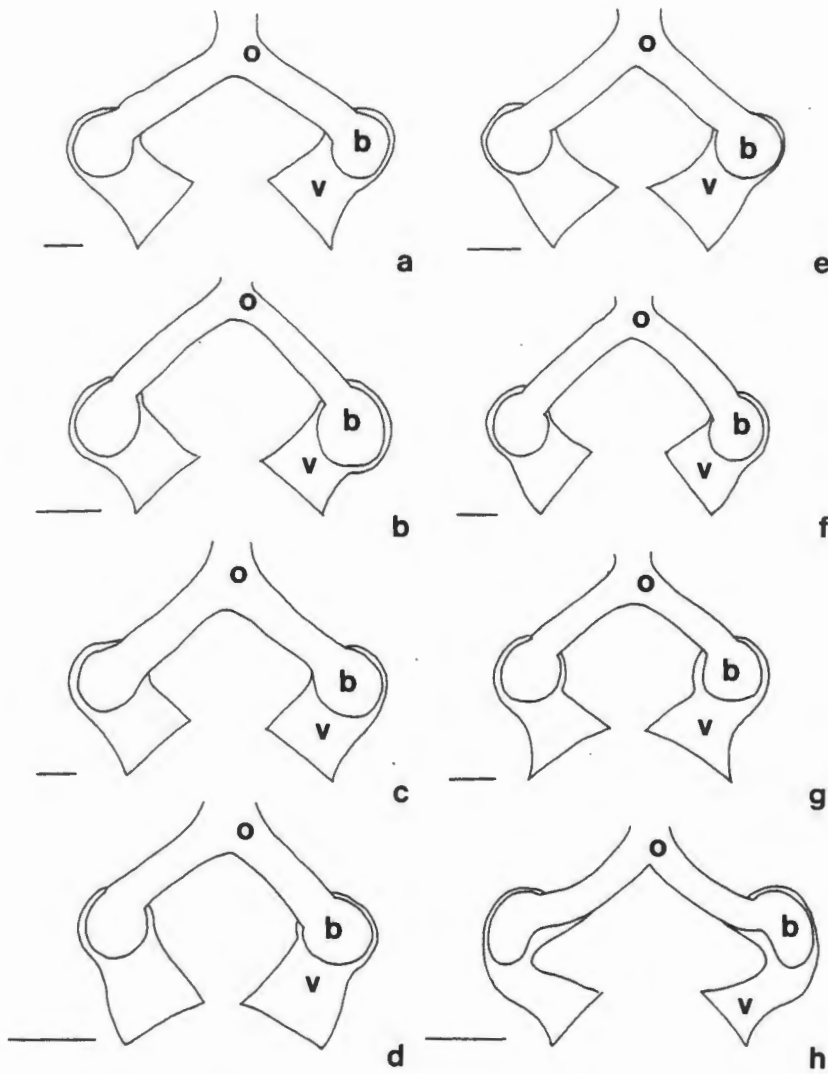


FIGURE 3.1 Diagrammatic representation of Spirostreptidae vulvae based on external topography. In all species examined bursae were located at the bottom of the vulval sacs. **a.** *Alloporus bilobatus*; **b.** *A. castanaes*; **c.** *A. flavifilis*; **d.** *A. levigatus*; **e.** *A. uncinatus*; **f.** *Orthoporoides pyrrocephalus*; **g.** *O. tabulinus*; **h.** *Triaenostreptus unciger*. Scale bars = 1mm.

o = oviduct, **b** = bursa, **v** = vulval sac.

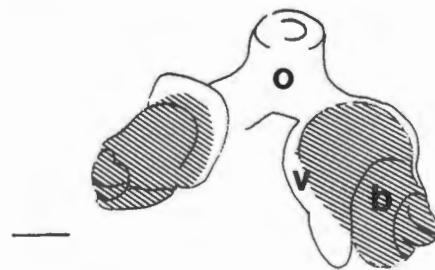


PLATE 3.2 Vulvae of *P. dipodontus*. b = bursa; o = oviduct; v = vulval sac (x14). Scale bar = 1mm.

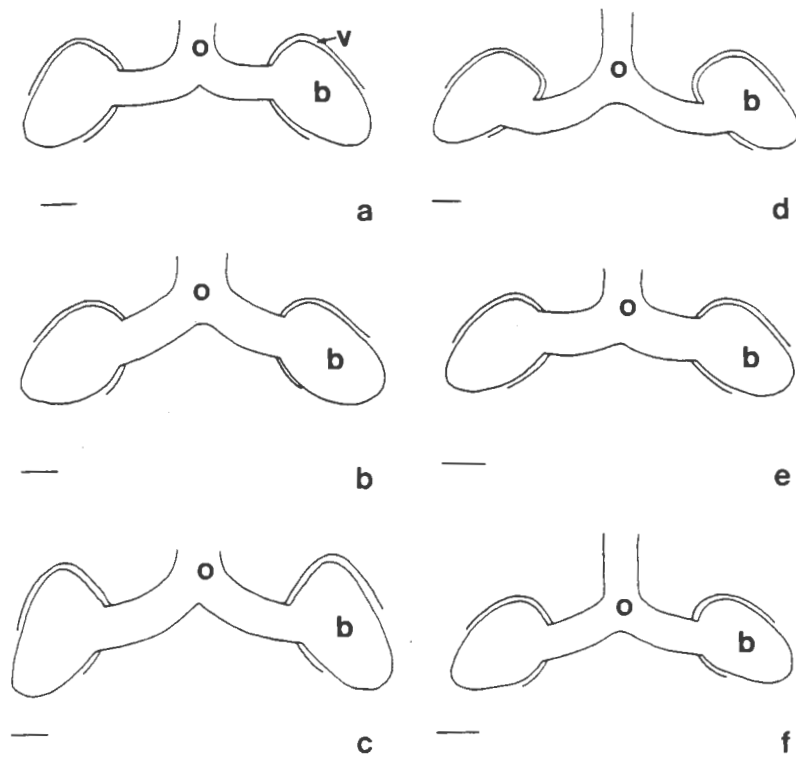


FIGURE 3.2 Diagrammatic representation of Harpagophoridae vulvae based on external topography. Note that in all species the bursae protrude from the vulval sacs. **a.** *Harpagophora levis*; **b.** *Poratophilus diplodontus*; **c.** *P. similis*; **d.** *Poratophilus* sp.1; **e.** *Poratophilus* sp.2; **f.** *Poratophilus* sp. 6. Scale bars = 1mm.
o = oviduct, **b** = bursa. The vulval sac is indicated on *H. Levis* only, where **v** = vulval sac.

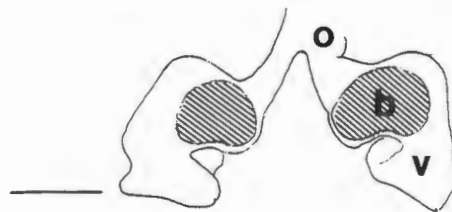


PLATE 3.3 Vulvae of *S. fiedleri complicans*. b = bursa; o = oviduct; v = vulval sac (x14). Scale bar = 1mm.

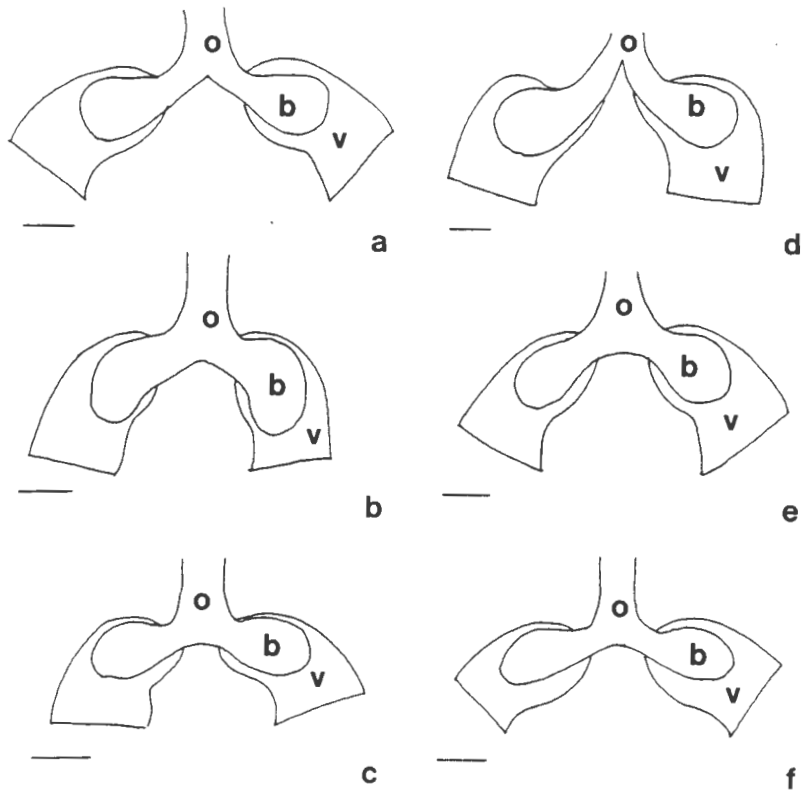


FIGURE 3.3 Diagrammatic representation of Odontopygidae vulvae based on external topography. In all species examined, bursae were situated at the bottom of the vulval sacs. **a.** *Chaleponcus hangklip*; **b.** *C. limbatus*; **c.** *Chaleponcus* sp.; **d.** *Spinotarsus fiedleri complicans*; **e.** *Spinotarsus* sp. 2; **f.** *Spinotarsus* sp. 3. Scale bars = 1mm, **o** = oviduct, **b** = bursa, **v** = vulval sac.

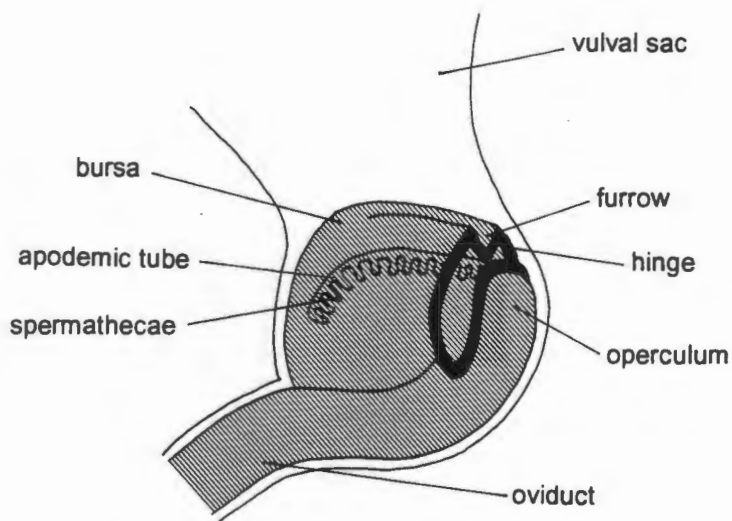


FIGURE 3.4 Generalised plan of the Spirostreptida bursa.

3.3.2 Bursal morphology

Bursal morphology is similar across all Spirostreptida families (Fig. 3.4). The **oviduct** passes into the **vulval sac** through the bursa. Its opening is covered by an **operculum** that is attached to the main body of the bursa via a chitinous **hinge**. This hinge comprises two thickened semicircles, one with an anterior kink, that articulate proximally. The bursa is the site of sperm storage, and sperm are stored in the **spermatheca**, a series of blind-ending **ampullae** that are connected via an **apodemic tube** to a **furrow** that runs along its anterior border. The apodemic tube arises at the lowest point in the anterior kink of the hinge, but does not communicate directly with the oviduct.

a. Spirostreptidae: *Alloporus uncinatus*

The bursa of *A. uncinatus* is located deeply within the vulval sac and is obscured from the gonopore by an internal invagination of the sac (Plate 3.1, Fig. 3.5a). It is roughly oval in shape, laterally compressed and covered by a valve on each side (Fig. 3.5b, Plate 3.4a). The valves are of different sizes and one wraps partially around the other. The cuticle that forms the valves is 17 - 22 μm wide.

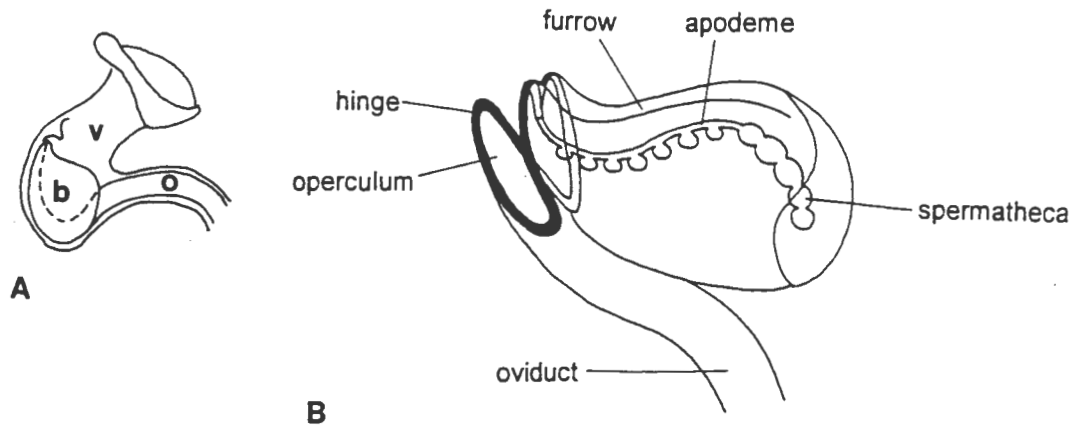


FIGURE 3.5 a. Diagrammatic representation of *A. uncinatus* bursa within the vulval sac. v = vulval sac, b = bursa, o = oviduct. b. *A. uncinatus* bursa.

The oviduct comprises simple columnar epithelium and is surrounded by striated muscles that run both transversely and longitudinally. At the anterior edge of the bursa, it enters the vulval sac from under the operculum, that is attached to the main bursal body via a hinge. One side of the hinge bears a kink and gives rise to a longitudinal furrow (240 μm deep, 630 μm long) that runs along the anterior border of the bursa (Plate 3.5). The membranes that form the folds of the furrow are 6 - 12 μm thick. The furrow gives rise to an apodemic slit (± 84 μm deep) which opens into the spermatheca. At the anterior end of the apodemic tube where it attaches to the hinge, a 'pin-cushion' (Tadler 1996) type of structure occurs within the cuticle (Fig. 3.6). It is probably analogous to the sensory hair ("*poil sensoriel*") described by Brölemann and Lichtenstein (1919). At the posterior limit of the apodemic slit, the spermatheca continues as a free undulating tube, ± 350 μm long (Plate 3.4b, Plate 3.5c). The spermatheca is 43 - 48 μm in diameter where it attaches to the apodemic slit. In the free tube in the posterior bursa it reaches up to 84 μm in diameter. Sperm were clearly visible (Plate 3.6a, b, c).

Striated muscles that stained bright red (Mallory) insert at the base of the apodemic slit and fan out to the bursal walls where they cross the epidermal layer and attach to the cuticle (Plate 3.5, Plate 3.6c). Muscles are surrounded by bursal glands that stained lilac (Mallory) and fill most of the bursal cavity. It is not clear where these glands open.

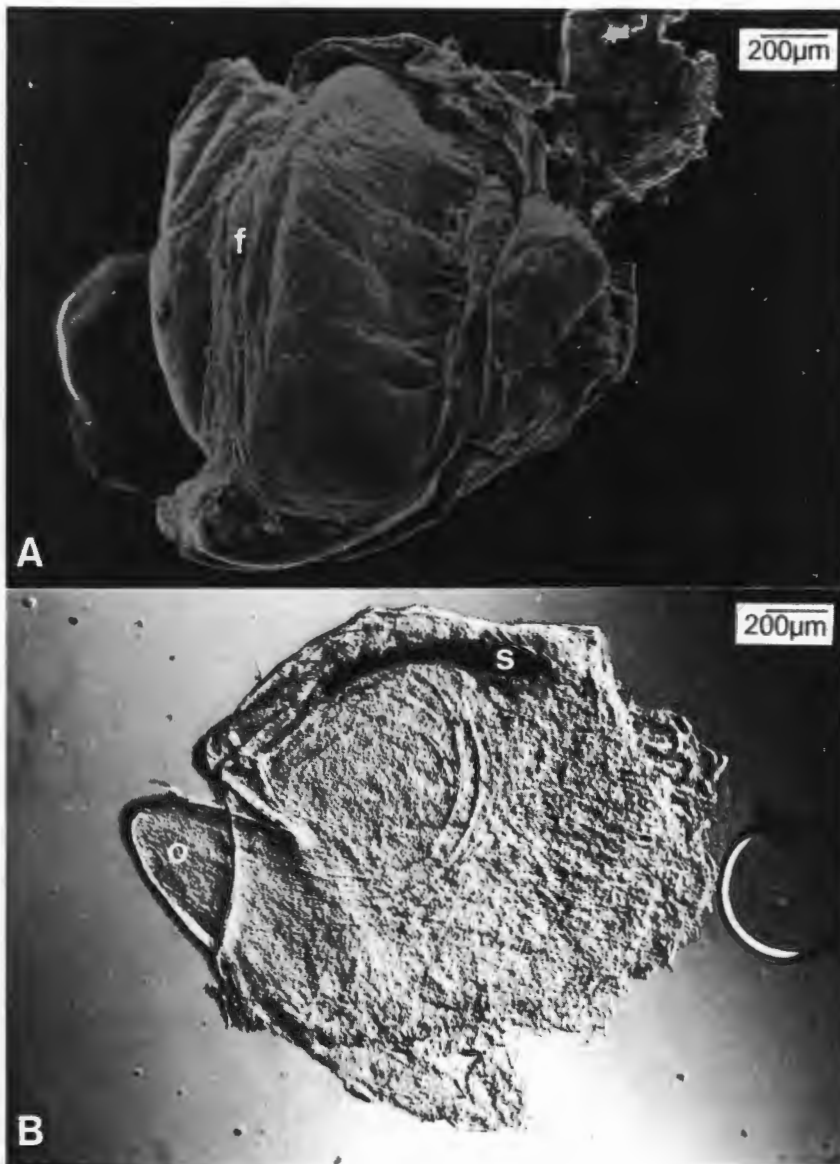
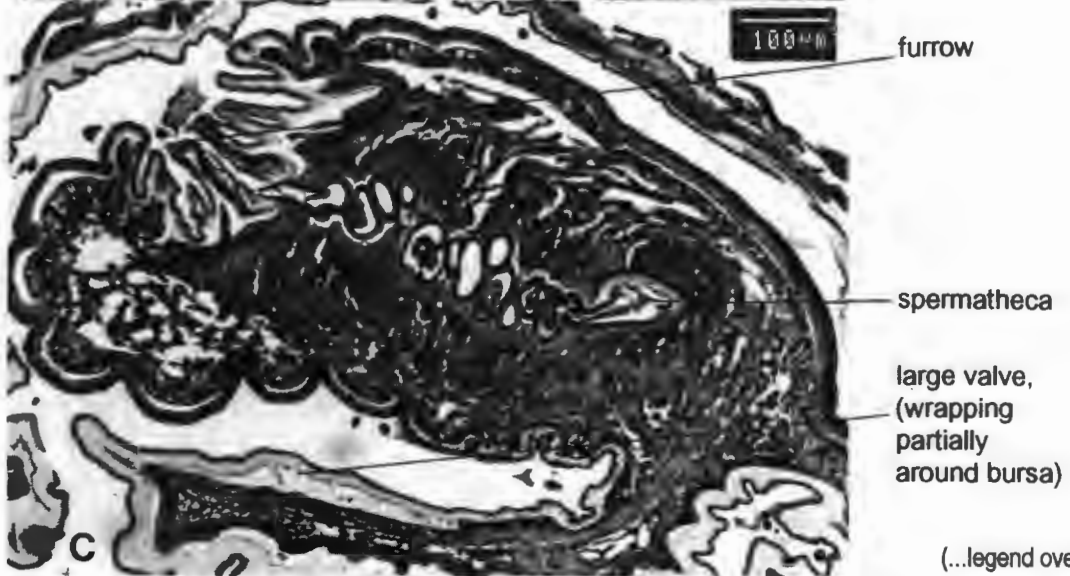
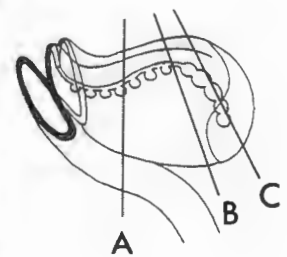
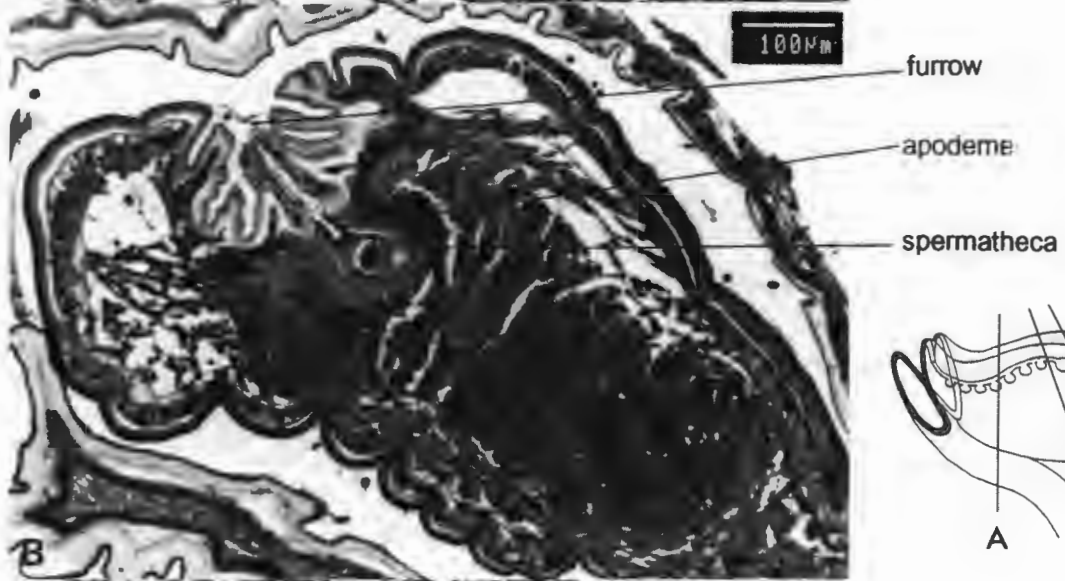


PLATE 3.4 A. *uncinatus* bursa a. SEM; b. Phase contrast light microscopy (x40). o = operculum; s = spermatheca; f = furrow.

The granules that are transferred with ejaculates (see Chapter 2) do not enter the spermatheca. In females dissected 24 and 48 hours post copulation, they were concentrated in the furrow, apparently blocking entry to the apodemic slit and the spermatheca (Plate 3.7a, b). Interestingly, not all females dissected soon after copulation had granules in their furrows.



(...legend overleaf)

PLATE 3.5 (from previous page) Section through *A. uncinatus* bursa at varying distances from the operculum.
a. close to operculum. Note oviduct passing under apodemic tube and muscles fanning from tube to bursal walls;
b. Section closer to midline, no longer crossing through the oviduct; **c.** Section down posterior end cutting through the free part of the spermatheca. Note the larger valve wrapping partially around the bursa in b & c (all x100).

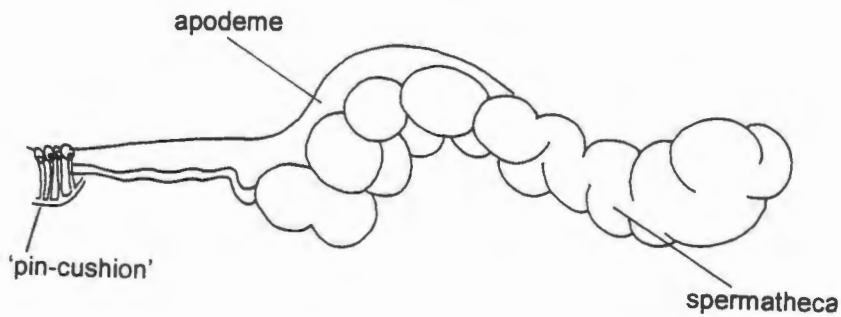
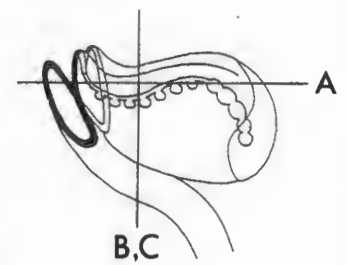
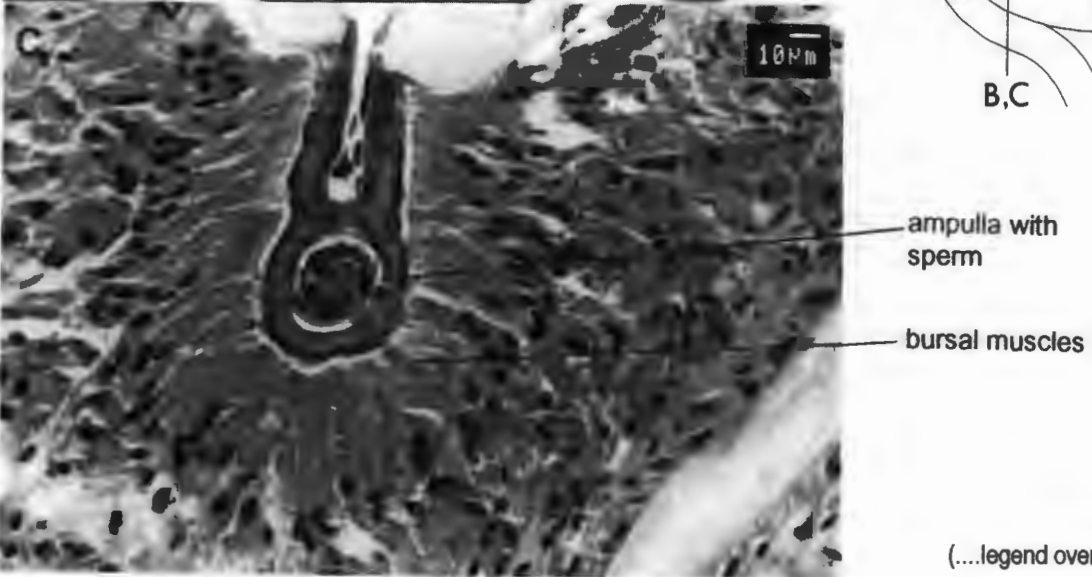
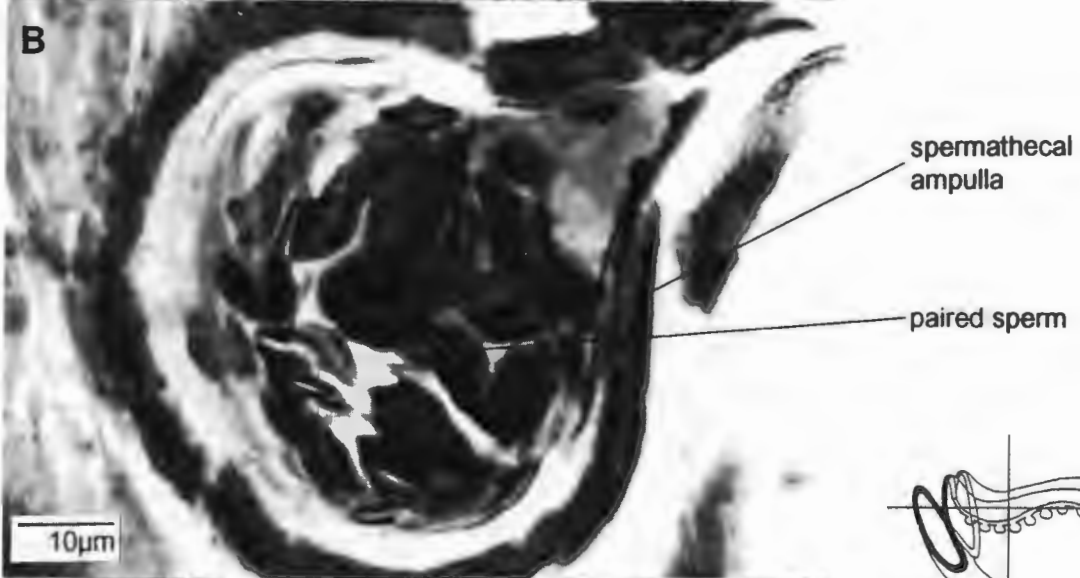


FIGURE 3.6 Diagrammatic representation of *A. uncinatus* apodemic tube (apodeme) and spermathecae. The pin-cushion type structure is located at the anterior end close to the point of oviduct insertion. Its function may be sensory.



(...legend overleaf)

PLATE 3.6 a. (from previous page) Section through the spermatheca (across the top of the bursa). Note bursal muscles and glands (x250); **b.** High magnification image of spermathecal ampulla (x1000). Note pairing of sperm; **c.** Section through furrow illustrating apodemic slit and spermatheca and associated glands and muscles (x400).

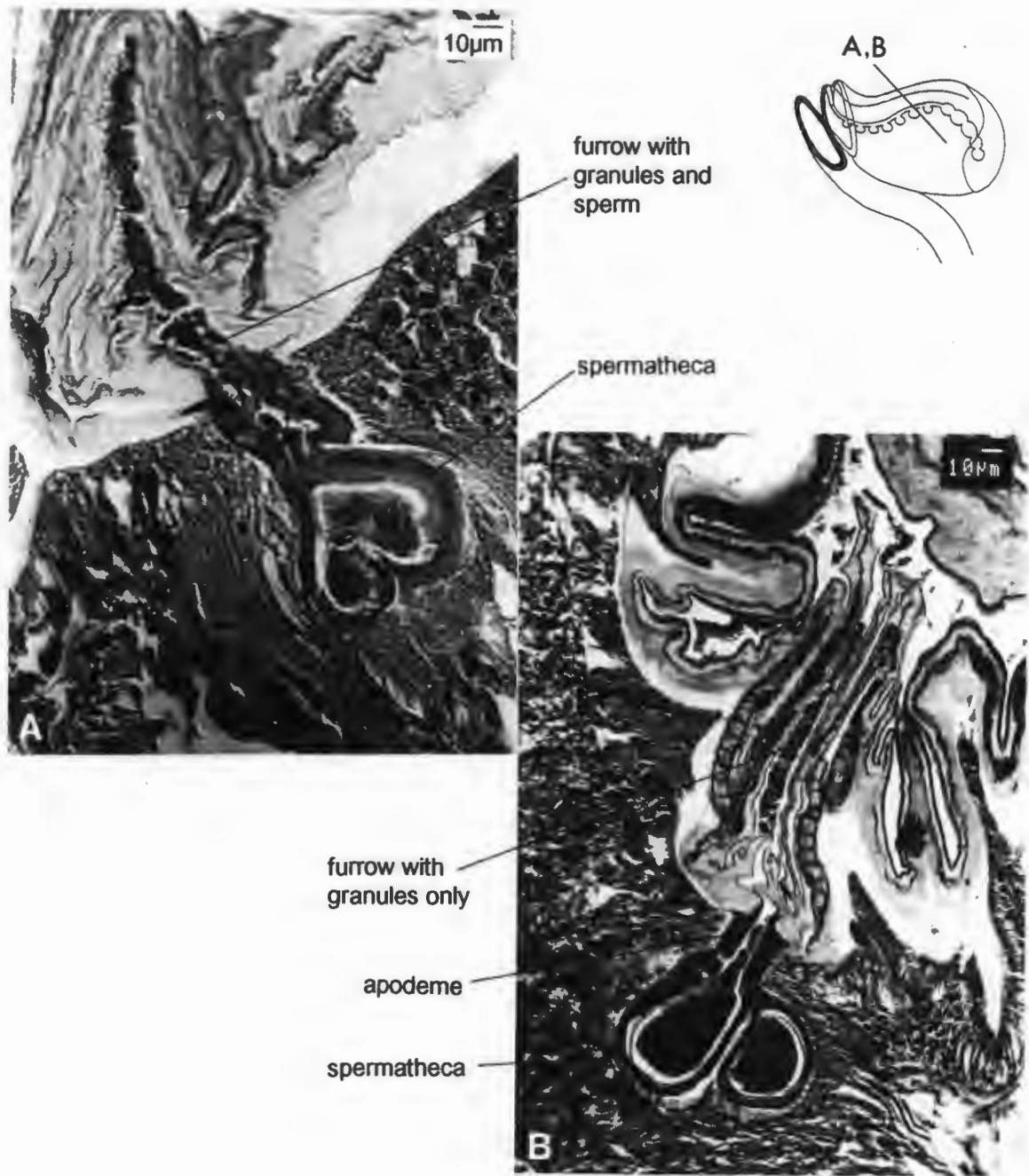


PLATE 3.7 Cross-section of *A. uncinatus* bursa removed 24 and 48 hours post copulation. **a.** 24 hours following copulation both sperm and granules occurred in the furrow, **b.** but only granules occurred 48 hours following copulation (both x250).

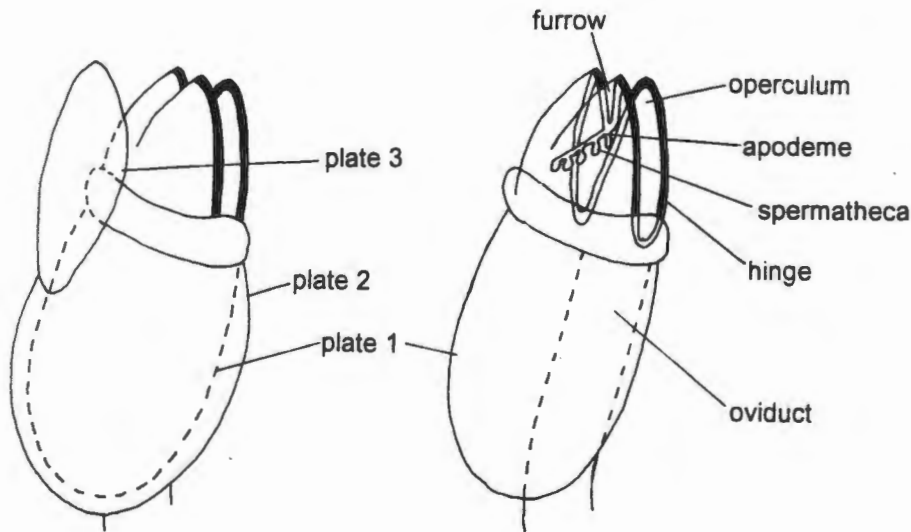


FIGURE 3.7 A. Diagrammatic representation of *P. diplodontus* bursa.
B. Detail of bursa (plates 2 and 3 removed).

b. Harpagophoridae: *Poratophilus diplodontus*

In *P. diplodontus* the bursa fills the vulval sac, and when it is severed from the body cavity, its distal end protrudes from the gonopore (Plate 3.2, Fig. 3.7, Plate 3.8). The distal end of the bursa is in close proximity to but does not protrude from the gonopore when it is in situ. As above, the bursa comprises two valves that are joined via a chitinous hinge to the operculum, and form a furrow that bears the sperm storage ampullae. The bursal cuticle is $\pm 80 \mu\text{m}$ thick, considerably thicker than that of *A. uncinatus* and *S. fiedleri complicans*, and this may be an adaptation to its protrusion from the gonopore. In addition to the valve system, the bursa bears three plates, two of which form the posterior body of the bursa and a hollow cavity through which the oviduct passes into the valve system. The third plate covers the gonopore. The valve system invaginates beneath plate 2 proximally, and striated muscles are associated with this invagination (Plate 3.9a, b).

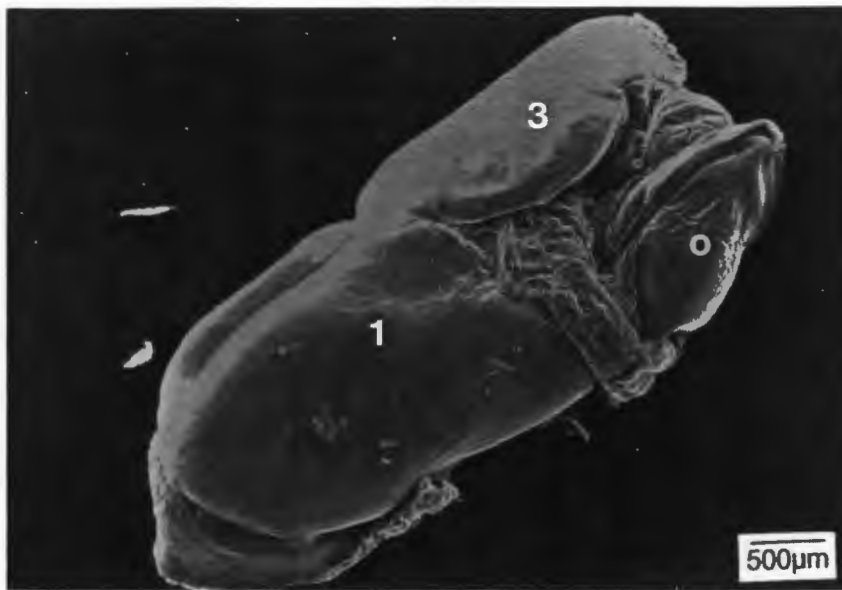


PLATE 3.8 *P. dipodontus* bursa with plate 2 removed (SEM). o = operculum; 1 = plate 1; 3 = plate 3.

The furrow that is formed by the valves is $\pm 349 \mu\text{m}$ deep and opens into the apodemic slit. As in *A. uncinatus*, the membranes that give rise to the slit resemble typical arthroal membrane, except where they are extensions of the hinge (Plate 3.9c, d). The slit is short ($\pm 388 \mu\text{m}$) and gives rise to the spermatheca which is 33 - 72 μm in longest diameter. The spermatozoa that occurred within the spermatheca were frequently paired (Plate 3.10a, b). Striated muscles attach at the bottom of the spermatheca and fan out to the bursal walls (Plate 3.10c). Bursal glands occur between these muscles in the proximity of the spermatheca. Their point of attachment was not clear.

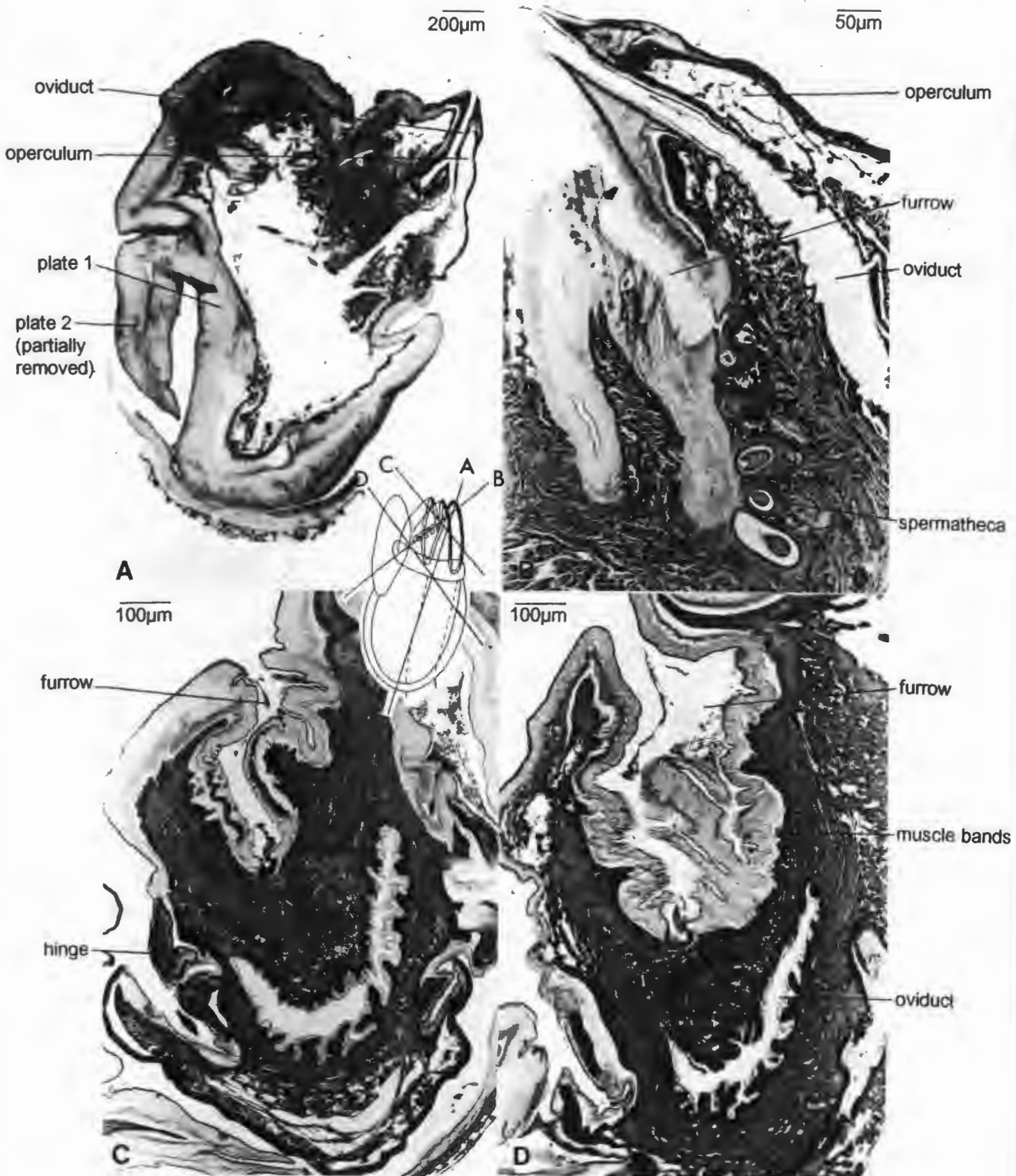


PLATE 3.9 *P. dipodontus* bursa. a. Section through whole bursa (x25); b. Section through the spermatheca (x100); c, d. Sections through the distal end of the bursa at varying distances from the operculum. Note hinges in c (x100).

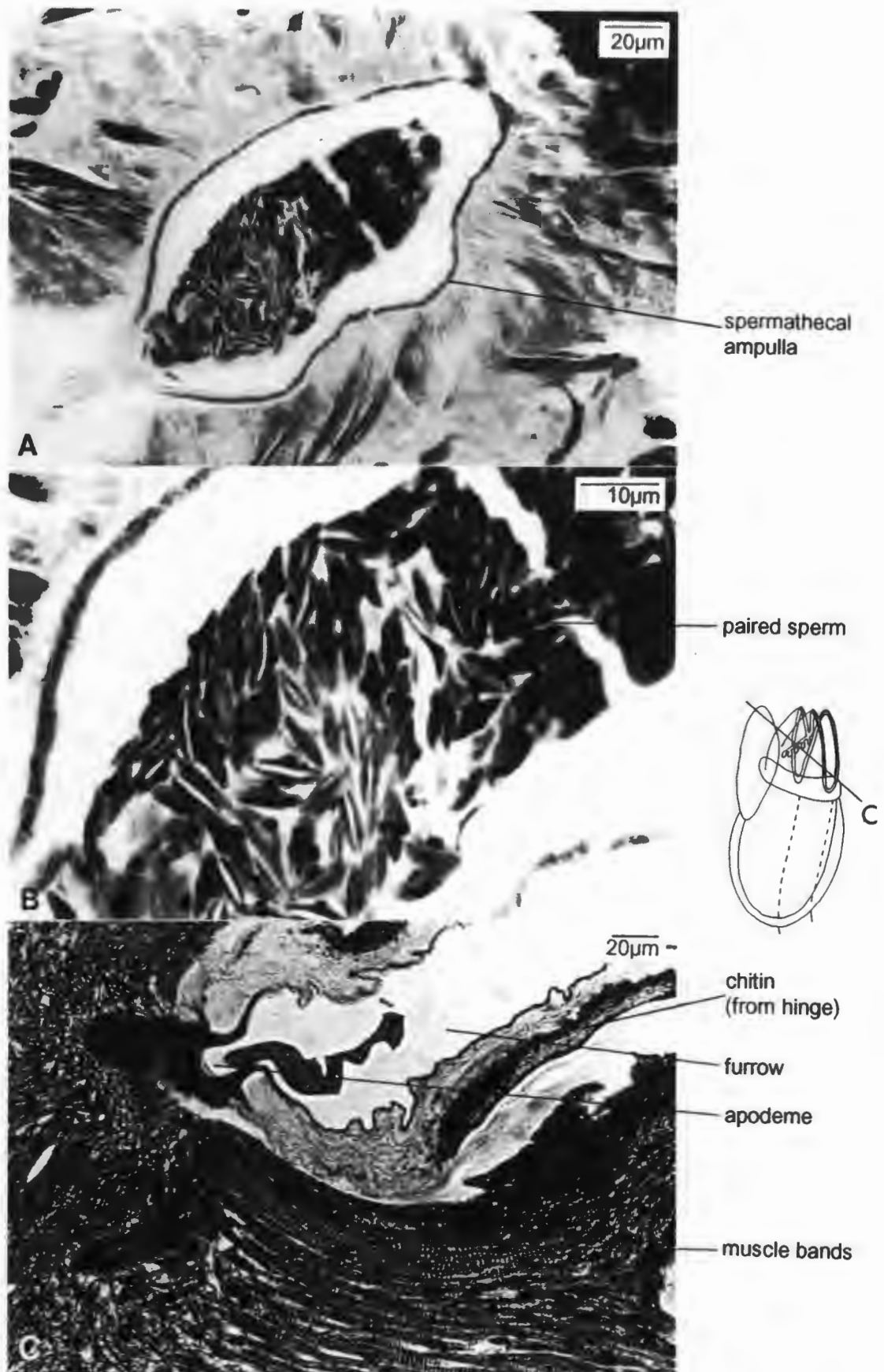


PLATE 3.10 a. Section through *P. dipodontus* spermatheca (x400); b. *P. dipodontus* sperm (x1000); c. Section through bottom of furrow illustrating muscle attachments to the apodeme (x250).

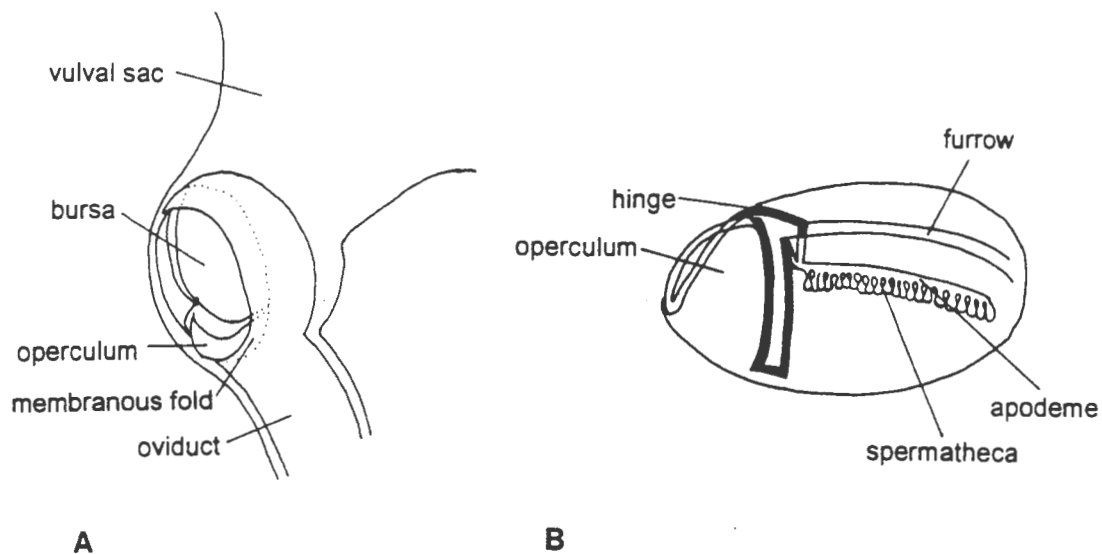


FIGURE 3.8 *S. f. complicans* bursa. a. Bursa within the vulval sac. b. Bursa.

c. Odontopygidae: *Spinotarsus fiedleri complicans*

As in *A. uncinatus*, in *S. fiedleri complicans* the bursa is located deeply within the vulval sac (Plate 3.3, Fig 3.8a). It lies within a membranous fold that is histologically similar to the vulval sac membrane (i.e. it resembles arthroal membrane: stains with aniline blue, overlies simple columnar epithelium, 20 - 115 μm wide) and obscures the bursa from the gonopore.

The bursa itself is dorso-ventrally compressed and its cuticle is 15 - 36 μm thick (Plate 3.11a, b). As in *A. uncinatus* and *P. diplodontus*, it comprises two valves and an operculum. These are joined by two hinges and articulate close to the bottom of the bursa. One of the hinges bears a kink that gives rise to the apodemic slit and spermatheca. The hinges that join the operculum to the bursa differ from those previously described in that the point of articulation on one side is widened. This modification widens the operculum opening and may be a necessary consequence of the dorso-ventral bursal compression. The oviduct emerges from beneath the operculum.

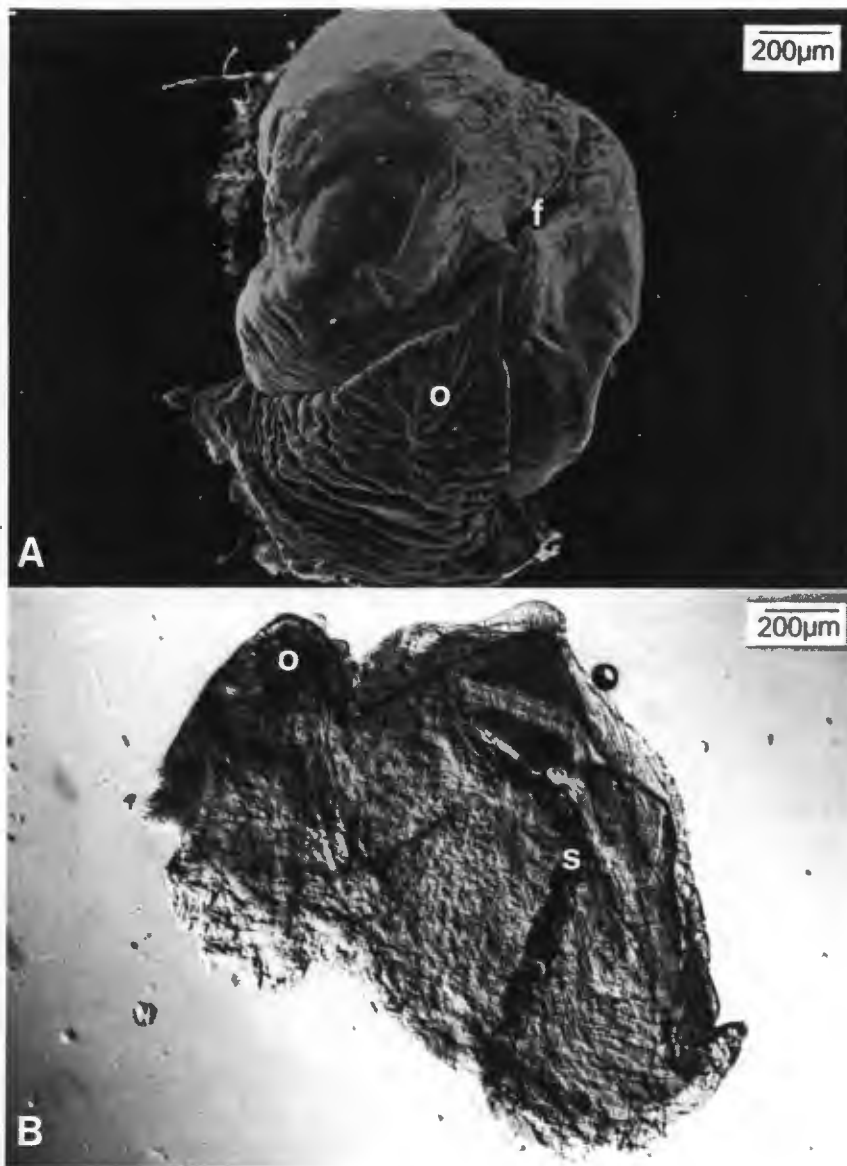


PLATE 3.11 *S. f. complicans* bursa. a. SEM. b. Phase contrast light microscopy (x40).

s = spermatheca; o = operculum; f = furrow.

The furrow that gives rise to the apodemic slit and the spermatheca is $\pm 200 \mu\text{m}$ deep (Plate 3.12a, b). Unlike both *A. uncinatus* and *P. diplodontus*, the furrow does not run horizontally along the midline of the bursa, but veers off to one side (Fig. 3.8; Plate 3.11a). The apodemic slit gives rise to a looped spermatheca in which sperm can be seen (Plate 3.11b; 3.13a, b). The spermatheca is $\pm 740 \mu\text{m}$ long and ranges from 36 - 72 μm in diameter. Sperm are paired (Plate 3.13c). Striated muscles that are interspersed with bursal glands fan out from the apodemic slit to the bursal walls (Plate 3.12b, 3.13b).

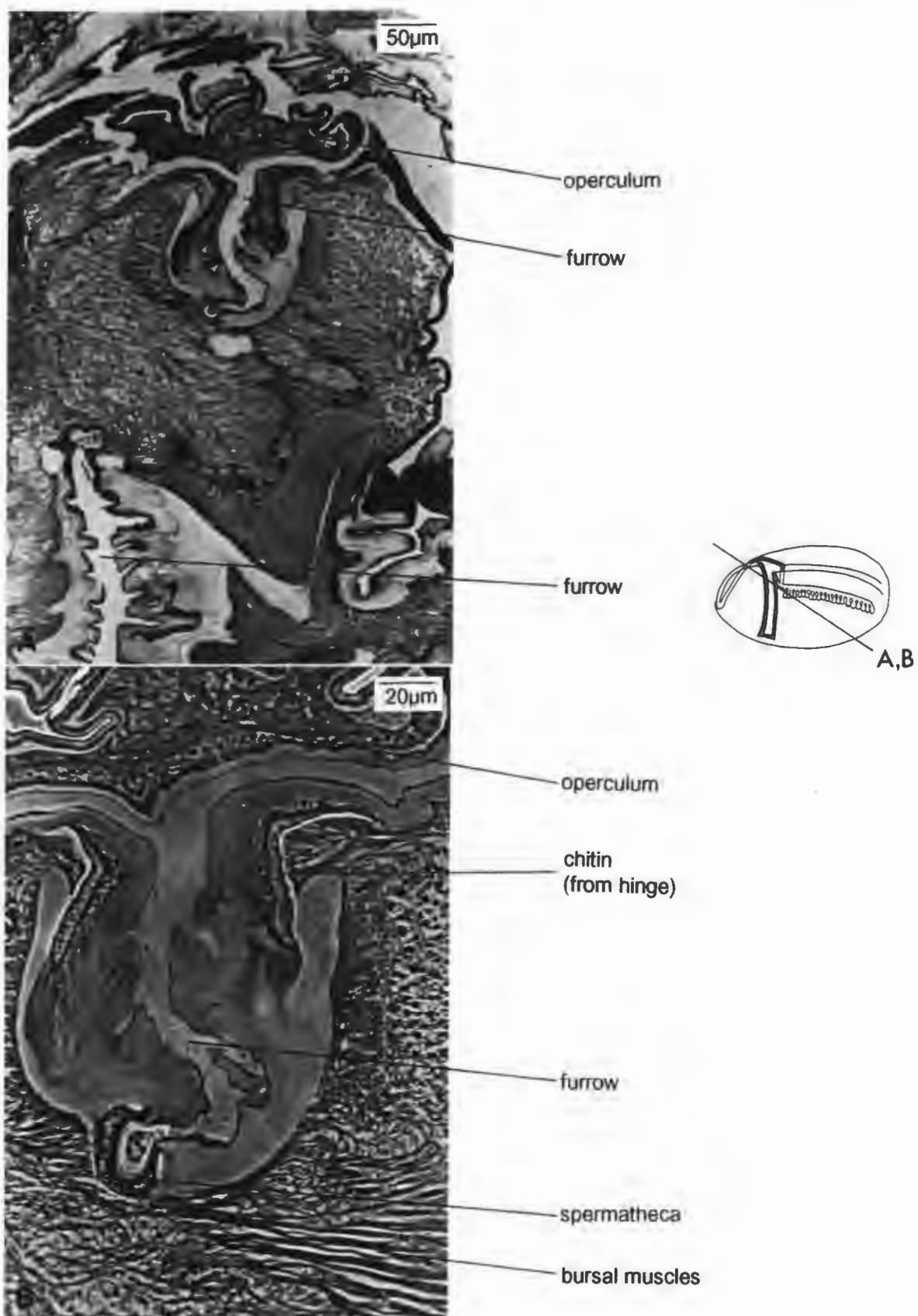


PLATE 3.12 Section through *S. f. complicans* bursa through the operculum. a. x100 b. x250.

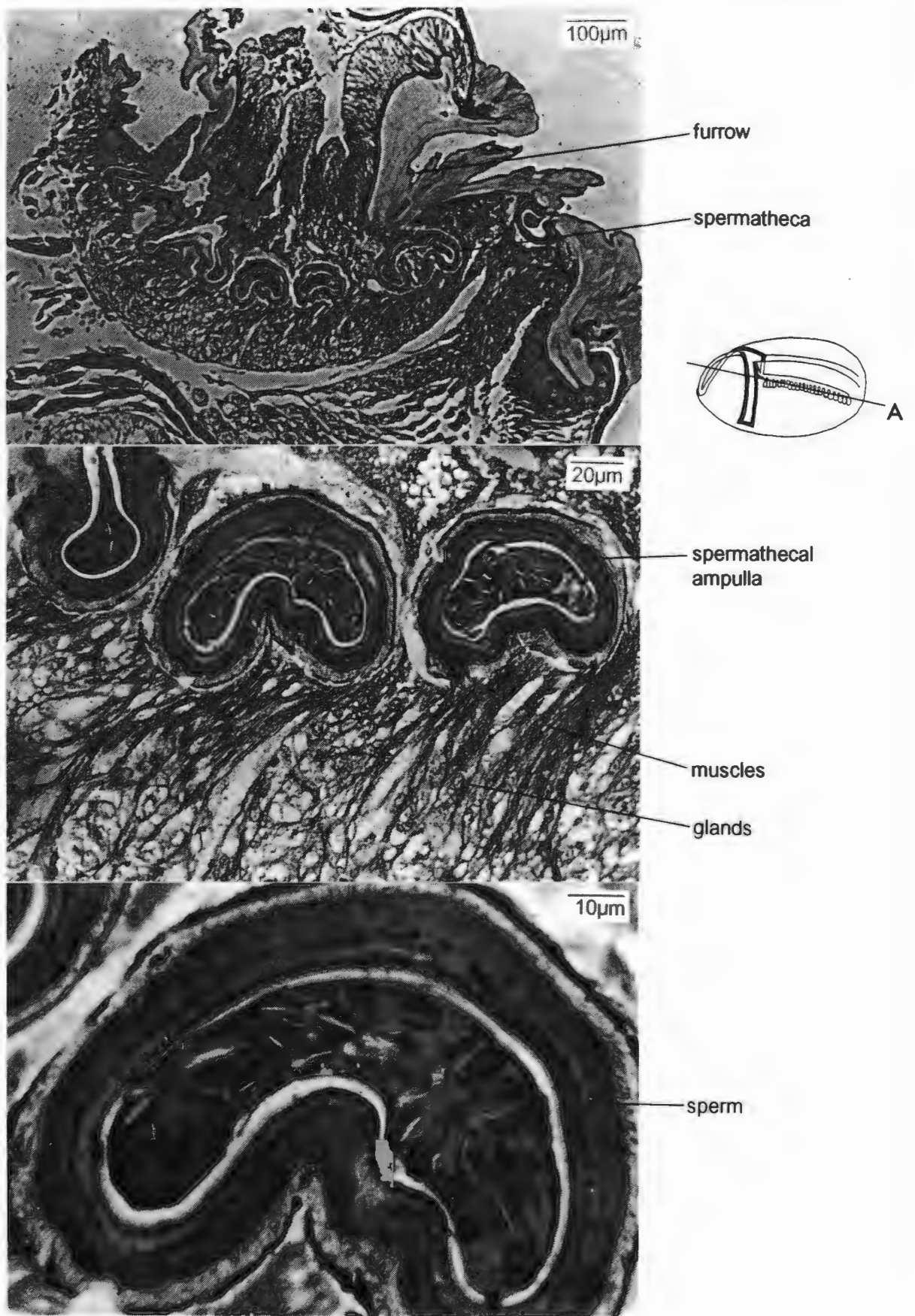


PLATE 3.13 *S. f. complicans* spermathecal detail. a. Section through spermatheca (x100). b. Detail of bursal muscles and glands that surround the spermatheca (x400). c. Section through spermathecal ampulla illustrating paired sperm (x1000).

3.2.3 Cuticles

At a cytological level, there was little difference between the structure of the tissues examined. Two basic types of cuticle, in which the principal layers were easily distinguished, comprised the vulvae of all three species (stain colours refer to Mallory's triple connective tissue stain).

a. The cuticle of the vulval sac membranes, the furrows, the posterior bursae of *A. uncinatus* and *S. fiedleri complicans* and the whole bursa except the operculum of *P. dipodontus* were formed from arthroal membrane. It comprised an epicuticle, an exocuticle that stained with aniline blue and an endocuticle that was either opaque or also stained with aniline blue. Aniline blue indicates a loose texture and is typical of arthroal membrane (Neville 1975). Arthroal membranes ranged from 57 - 187 μm and the thicker areas, in particular those constituting the vulval sac membranes, were probably unstretched regions. The lamellae that typify arthroal membrane were easily distinguished (Plate 3.14a).

b. The hinges, the apodemic tubes and the spermathecae of all three species, the anterior bursae of *A. uncinatus* and *S. fiedleri complicans*, and the operculum of *P. dipodontus* were formed from tanned solid cuticle. It comprised an epicuticle, and endo- and exocuticles that stained with aniline blue and an acid-fuchsin staining (red) mesocuticle (Richards 1951). Acid fuchsin indicates the presence of a rigid structure that is typically chitinous.

Both cuticle types overlie either simple columnar epithelium or glandular epithelium (parts of the bursae, particularly where the cuticle was solid), and a basement membrane (Plate 3.14a, b). Pore canals from the glandular epithelium cross into the endocuticle.

The surfaces of the vulval membranes of all three species resembled those of *O. pyrhocephalus* (Plate 3.15). They comprised small overlapping polygonal plates. The plates that constituted the bursa of *P. dipodontus* resembled the surface of the vulval walls (Plate 3.16). The walls of the bursae themselves were smooth and featureless in all species examined.

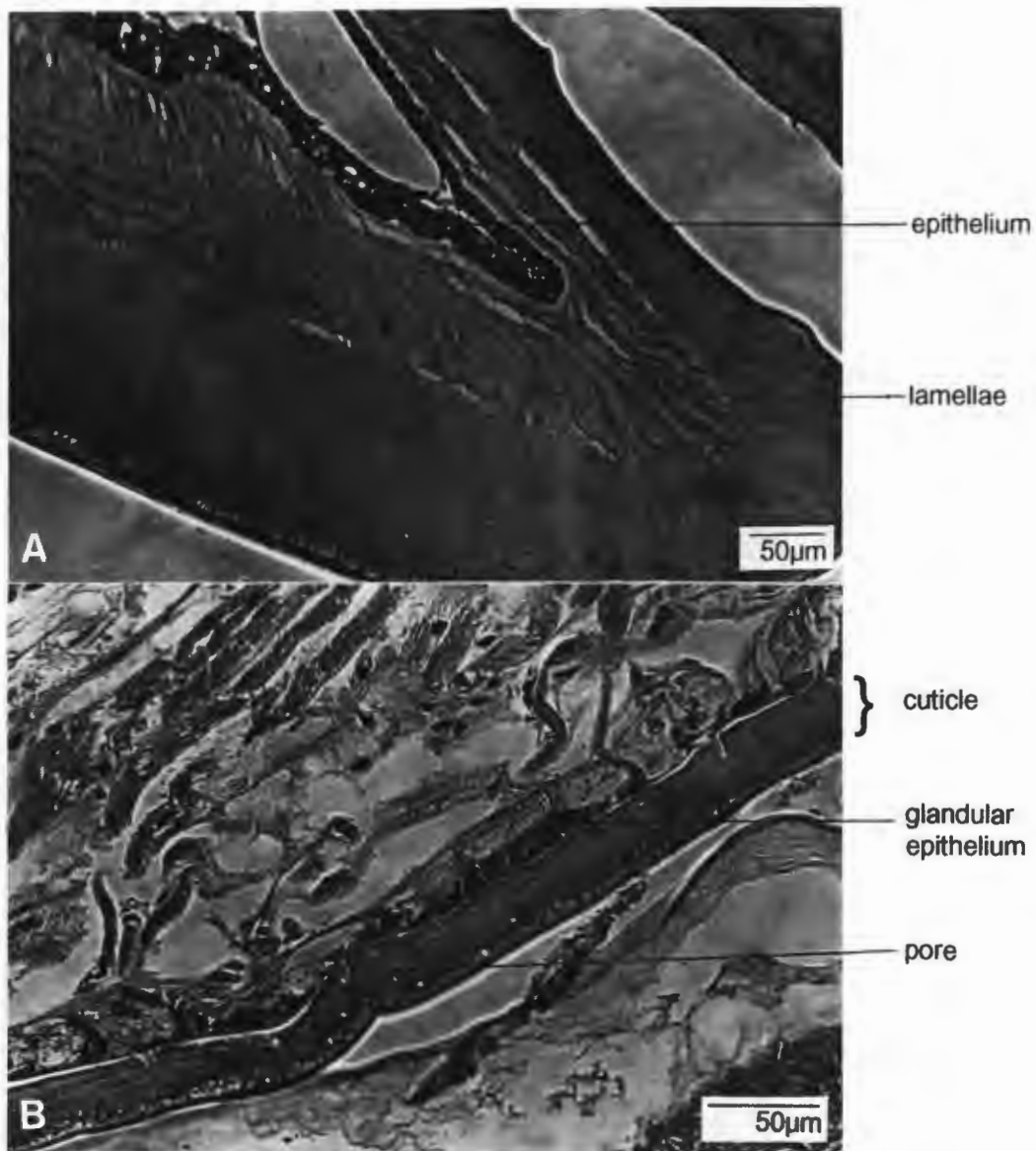
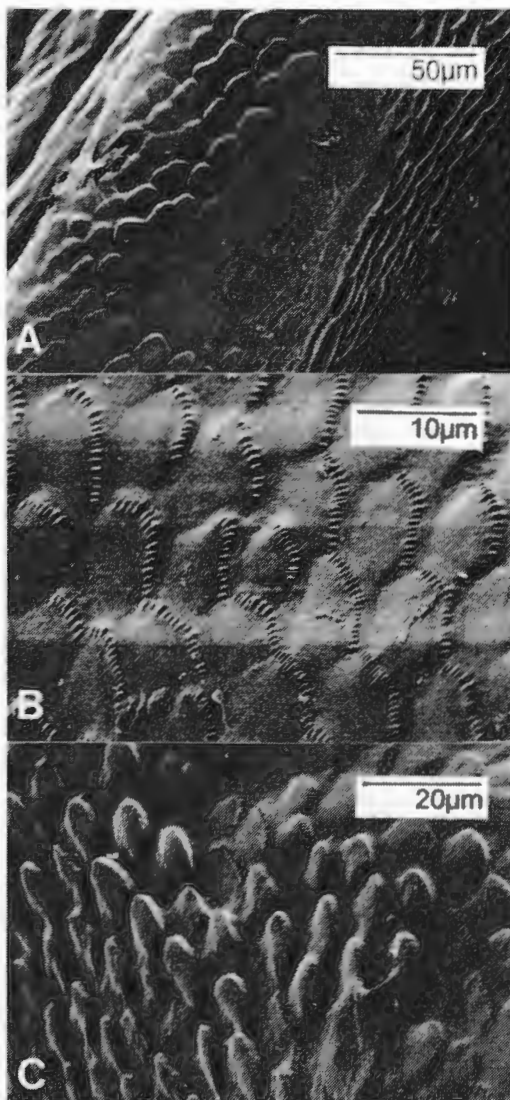


PLATE 3.14 a. Cuticle from plate 3 of *P. dipodontus* bursa (x250) b. Cuticle of *A. uncinatus* from anterior bursal valve (x400).

No sensory receptors could be detected with light microscopy in any of the species examined. The limitations of this technique are noted, and it is suggested that future work employ TEM to look for sensory structures, including the pin cushion structure at the anterior apodemic tube?



PLATES 3.15 The surface area inside *O. pyrhocephalus* vulval sacs (SEM, after Barnett 1991).

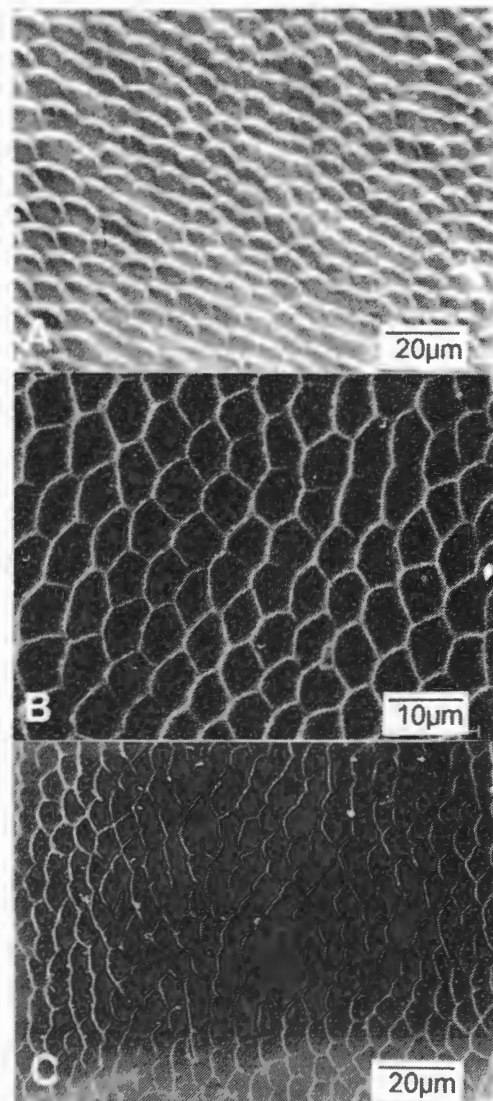


PLATE 3.16 The surface area of *P. dipodontus* bursal plates (SEM). A, Plate 2; B, C Plate 3

3.4 Discussion

While subtle morphological differences can be distinguished at the species level and are commonly used taxonomic characters (e.g. Kurnik and Thaler 1985), the general form of the vulvae is conserved at the family level (Figs. 3.1, 3.2, 3.3). Accordingly, the histological analyses of the bursae of *A. uncinatus*, *P. diplodontus* and *S. fiedleri complicans* are taken to represent the Spirostreptidae, Harpagophoridae and Odontopygidae respectively.

3.4.1 Movement and storage of ejaculates

a. Movement of ejaculates into the stores

Although sperm are placed directly in the spermathecae of some arthropod species (Bonhag and Wick 1953; Eberhard 1996), in most species the actual sites of sperm storage are not accessible to male genitalia (e.g. Smith 1984; Eberhard 1985; 1996; Eady 1994a, Siva-Jothy and Hooper 1995). Consequently sperm often change their position within the female tract. Sperm movement can be effected by the sperm themselves (Nonidez 1920 cited in de Wilde and de Loof 1973; Rockstein 1973; Sivinski 1984) but more often, and especially where sperm are non-motile, this is a consequence of female processes (Linley and Simmons 1981; Birkhead and Moller 1992; Kaulenas 1992; Eberhard 1996). Muscles associated with the female tract often play a role in sperm transport (Chapman 1969; Villavaso 1975a; Walker 1980; Linley and Simmons 1981; 1983; Eberhard 1985; 1991; Heming-van Battum and Heming 1986; Gomendio and Roldan 1991; Otronen and Siva-Jothy 1991; Birkhead and Moller 1992; Eady 1994b; Rodríguez 1994). It has been suggested that naturally selected systems of sperm translocation could subsequently be used to selectively store ejaculates of different males (Eberhard 1996). Cryptic female choice based on male body mass and "copulatory vigour" results in differential sperm storage in the Sierra dome spider *Linyphia litigiosa* (Watson 1990; 1991; Watson and Lighton 1994).

Spirostreptida sperm are non motile. Because males generally do not have direct access to the spermathecae (see later), it has been suggested that sperm movement into the stores is female mediated. Where bursal muscles apparently do not attach to the spermathecae, it has been suggested that the bursal epithelia suck fluid (and sperm) into the bursae (*Craspedosoma* spp., Tadler 1993). In the three Spirostreptida species

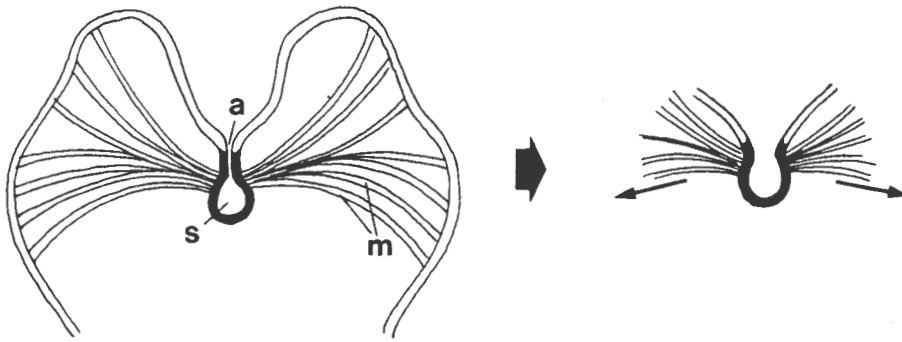


FIGURE 3.9 Diagrammatic representation of association of bursal muscles, apodeme and spermatheca. Apodeme opens up with bursal muscle contraction. **s** = spermatheca; **a** = apodeme; **m** = bursal muscles.

examined in this chapter, bursal muscles attach to the apodeme and individual ampullae, and fan out to the bursal walls. This arrangement is similar to that of the Polydesmida in which sperm are believed to enter the individual ampullae via pumping contractions of the bursal muscles (Seifert 1932). Contraction would cause the ampullae to open up, allowing ejaculates in the vicinity of the furrow to enter them (Fig. 3.9). The regulation of sperm movement through these muscle movements may also facilitate selective storage of ejaculates: based on syncopulatory mate assessment (as in Watson 1991), females could selectively store ejaculates.

b. Selection between stored ejaculates

The incidence of multiple sperm stores has also been suggested to be a mechanism by which females control paternity post insemination, because by sorting ejaculates into different compartments females may be able to choose between them (Sivinski 1984; Ward 1993; Eberhard 1996). The spermathecae of the Spirostreptida bear several interconnected ampullae. It is not clear how these divisions would affect the ability of females to select between ejaculates of different males.

c. Prolonged storage

Sperm were found in the spermathecae of an *A. uncinatus* female 12 months after copulation, indicating that, as in many arthropods (Parker 1970a; McVey and Smittle 1984; Smith 1984; Romoser and Stoffolano 1994) including Polydesmid millipedes (Snider 1984), storage over long periods is possible. The ability of females to store

sperm for protracted periods further uncouples processes of fertilisation from the presence of males.

Although not necessarily (Eberhard 1996), prolonged storage of sperm has been associated with specialised cells and nutrient provision (Michener 1974; Kaulenas 1992). This may be the function of the bursal glands that always occur in close association to the ampullae (Brölemann and Lichtenstein 1919; Demange 1988). Brölemann and Lichtenstein (1919) believed glandular secretions to be triggered by the sensorial hair ("*poil sensoriel*") that occurs at the extremity of the oviduct-side of the apodemic tube.

3.4.2 Mechanisms of sperm competition

a. Stratification

During oviposition, ova enter the vulval sac from beneath the operculum and pass over the anterior bursae. The ampullae are blind ending and ejaculates from successive males could be layered. This implies that sperm competition via ejaculate stratification cannot be excluded (Walker 1980). However, in taxa in which stratification is believed to account for high P_2 values, P_2 has been shown to decline with time due to subsequent ejaculate mixing (e.g. the dragonfly *Erythemis simplicicollis* McVey and Smittle 1984; the damselfly *Mnais pruinosa pruinosa*, Siva-Jothy and Tsubaki 1989; the harlequin beetle-riding pseudoscorpion *Cordylochernes scorpioides*, Zeh and Zeh 1994). Given the probable temporal delay between insemination and oviposition in the Spirostreptida, it is equally possible that ejaculates stored within the spermathecal ampullae mix prior to fertilisation, especially given the lack of mobility in the sperm of these animals. It is noted that hypotheses regarding the mechanisms of sperm mixing are speculative. Due to low Reynolds numbers, the medium in which sperm are suspended is probably viscous. It is not clear how pumping or other mechanisms would actually mix such viscous fluids'

b. Displacement

The evolution of gonopod complexity was discussed in Chapter 2 and function was inferred from both morphology and functional morphology, but without considering the action of the gonopods within the vulvae. It was concluded that (among other things)

telopodites could function in ejaculate placement, repositioning and displacement, and female stimulation. All of these are dependent on the syncopulatory association of gonopods and vulvae. Likewise, the significance of the positioning of the bursae within the vulvae cannot be appreciated without cognisance of their syncopulatory relationship with the gonopods.

In the other Diplopoda orders in which genital function has been studied, bursae are situated in shallow vulval sacs (Julida, Tadler 1996; Chordeumatida, Tadler 1993; Spirobolida, pers. obs.) and during copulation are pulled from the vulvae by the gonopods (Chordeumatida: Tadler 1993; Julida: Haaker and Fuchs 1970; Tadler 1996). Chordeumatida gonopods and bursae juxtapose during copulation and brushes on the anterior gonopods transfer sperm to the spermathecae by partially entering them (Haaker 1971; Tadler 1993). These brushes may also function in sperm displacement (Tadler 1996).

In the Julida, gonopods and vulvae are not juxtaposed, but each gonopod bears a flagellum that reaches the bursal furrow through the distal tip of the posterior gonopods (Mauriès 1969; Tadler 1996). Both the syncoxite brushes (Chordeumatida) and the flagellae (Julida) have been suggested to function in sperm displacement (Tadler 1996).

In the Spirostreptida, the vulval sacs are formed by deep invaginations and bursae are not everted during copulation. The position of the bursae within these sacs differs between families. In the Spirostreptidae and Odontopygidae, the bursae are located deeply within the vulval sacs. In the Harpagophoridae, the distal ends of the bursae protrude from the gonopore. These differences may have implications for mechanisms of sperm transfer and displacement: bursae located in close proximity to the gonopores may sanction direct male manipulation of ejaculates within the stores.

A preliminary investigation into the syncopula positions of the genitalia, using dissections of freeze-dried copula-pairs, showed the gonopod telopodites of the Spirostreptida and Odontopygidae to enter the vulval sacs (Fig. 3.10), and their distal telopodites may reach the anterior bursae. However, due to the orientation of the bursae and the configuration of the distal telopodite (in most species), it is unlikely that they enter the spermathecae. This finding is contrary to some earlier reports and conjecture (Gerhardt 1933; Kraus 1968; Barnett 1991; Barnett *et al.* 1995), but

consistent with that of Demange (1959, see Fig. 3.11) who concluded that the distal telopodites reach the distal bursae but do not enter them.

Because gonopods have no access to the sperm stores in the Odontopygidae and Spirostreptidae, direct ejaculate removal from the spermathecae is not mechanically possible (also see Parker 1970a; Walker 1980; Knowlton and Greenwell 1984; Waage 1986a). This does not preclude ejaculate displacement via flushing or direct removal from the vulval sacs. In the damselfly *Calopteryx splendens xanthostoma* (Siva-Jothy and Hooper 1995), and the bruchid beetle *Callosobruchus maculatus* (Eady 1994b), males have access to "temporary" store sites (the bursa in *C. s. xanthostoma*, the oviduct in *C. maculatus*), but not the spermathecae. In the mealworm beetle *Tenebrio molitor* (Gage 1992), the yellow dung fly *Scathophaga stercoraria* (Ward 1993) and the spruce budworm *Choristoneura fumiferana* (Retnakaran 1974), changes in P₂ are thought to be due to the migration of ejaculates from accessible regions to those that the second male's genitalia cannot reach.

In the Harpagophoridae, the bursae protrude from the gonopore and although telopodites are relatively shorter than those of the Spirostreptidae, they may have direct access to stored ejaculates. Male access to sperm stored in the vulvae may be subject to the configuration of the bursal muscles - with the muscles contracted, the bursal furrow would open up to facilitate displacement (either directly via the gonopods or indirectly via ejaculate flushing). In this way female control of the bursal muscles may sanction "selective surrender" (*sensu* Eberhard 1996) to sperm displacement.

c. Copulatory plugs?

Adaptations to sperm competition include mechanisms to prevent the pre-emption of previous ejaculates (Parker 1970a). The location of granules in the furrow but not in the apodemic tube or spermatheca of *A. uncinatus* females examined 24 and 48 hours post copulation suggests that they may block access through the apodemic tube and thereby function as postcopulatory plugs (see Thornhill and Alcock 1983). Granules could thus represent a conflict of interest because their presence may prevent females from storing the sperm of future males. The bursal furrows of some *A. uncinatus* females were not associated with granules following copulation, and this may relate to selective storage of ejaculates by females: if females do not contract the bursal muscles to allow sperm to enter the spermathecae, it may not be possible for granules to

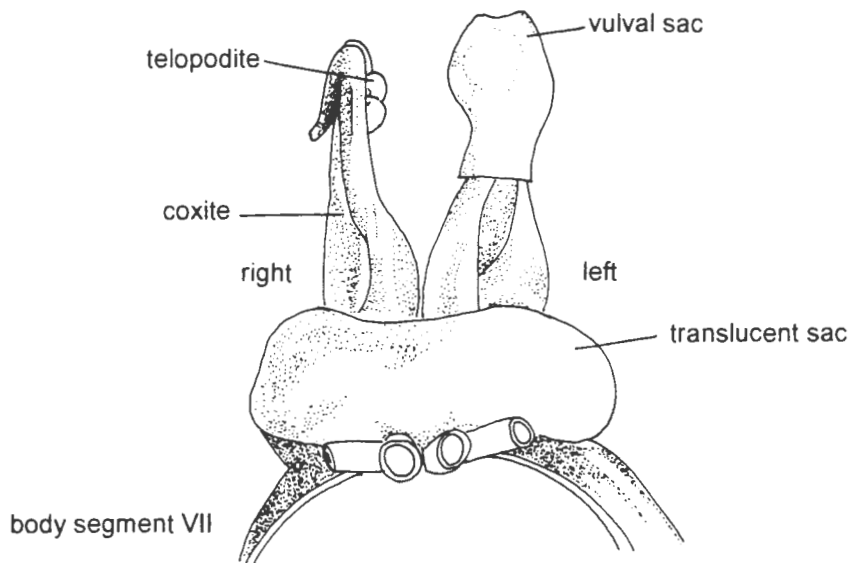


FIGURE 3.10 Aboral view of the syncopula position of the gonopods and vulvae of *O. pyrrocephalus* (Spirostreptidae), as exposed during the dissection of a copula pair that had been frozen in liquid nitrogen and freeze dried. The left gonopod is positioned within the vulval sac of the female. The vulval sac has been removed from the right gonopod and the telopodite is visible. It is fully retracted so that the distal plate touches the gonopod coxite. (see Fig. 2.21 for a description of *O. pyrrocephalus* gonopods)

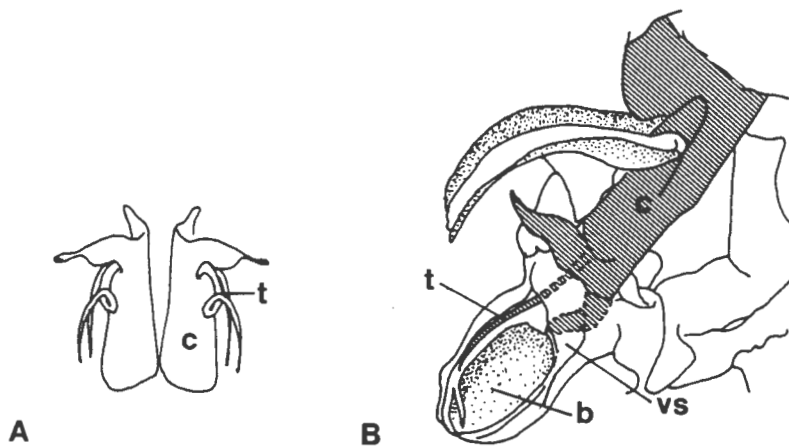


FIGURE 3.11 **A.** *Archispirostreptus tumuliporus* (Spirostreptidae) gonopods. **B.** Position of gonopods and vulvae during copulation. **c** = coxite; **t** = telopodite; **b** = bursa; **vs** = vulval sac. After Demange 1959.

accumulate in the furrow and apodemic tube either. It is not clear how the stratification of sperm and granules is achieved.

Granules were not found in the furrows of either *P. diplodontus* or *S. fiedleri complicans*. This may be due to selective storage by females (as above). Alternatively, this may indicate that only male Spirostreptidae produce granules for use as mating plugs. The latter option is consistent with the relative abundance of granules in Spirostreptidae ejaculates compared to those of the Odontopygidae and Harpagophoridae (see Plate 2.1).

3.4 Summary

In all three Spirostreptida families bursae are located in deeply invaginated vulval sacs. Sperm are stored in the bursae in spermathecae. These comprise a series of interconnecting ampullae and are associated with bursal glands and muscles. Muscles fan out from the spermathecae to the bursal walls. Bursal muscles may "sanction" cryptic female choice via control of ejaculate storage and manipulation. The distance of the bursa from the gonopore in the Odontopygidae and Spirostreptidae may preclude direct removal of rival ejaculates. In *A. uncinatus* (Spirostreptidae) females store sperm for protracted periods and the non-gametic component of the ejaculate, the granules, may function as mating plugs.

CHAPTER FOUR

THE TIMING OF INSEMINATION IN *ALLOPORUS UNCINATUS* (SPIROSTREPTIDAE) AND *PORATOPHILUS DIPLODONTUS* (HARPAGOPHORIDAE)

4.1 Introduction

The timing of insemination differs between and within taxa. It can be continuous over the entire duration of copulation, or instantaneous at the onset of copulation, following a pre-insemination phase, or at the end of copulation (e.g. continuous insemination: the cricket *Gryllus bimaculatus* Simmons 1986; the milkweed leaf beetle *Labidomera clivicollis clivicollis*, Dickinson 1986; the melon fly *Bactrocera cucurbitae*, Yamagishi and Tsubaki 1990; and see Eberhard 1996; at the onset: the spider *Phidippus johnsoni*, Jackson 1980 cited Eberhard 1996; following a preinsemination phase: *Drosophila melanogaster*, Gilbert *et al.* 1981; spiders, Austad 1984; the water strider *Gerris remigis*, Rubenstein 1989; the stalk eyed fly *Cyrtodiopsis whitei*, Lorch *et al.* 1993; towards the end of copulation: *Glossina* flies, Wall and Langley 1993). In some taxa, it has also been shown to vary (e.g. *Drosophila* spp., Grant 1983; the black fly *Simulium decorum*, Linley and Simmons 1983).

In Julida millipedes (see Appendix A), copulation comprises two distinct phases, and ejaculate transfer occurs during the second. During the pre-insemination phase, the flagellum on the gonopods moves rhythmically (Haaker and Fuchs 1970; see Fig. 2.1c). These movements have been implicated in female stimulation (Eberhard 1985), bursal cleaning (Haaker and Fuchs 1970) and both direct and indirect sperm displacement (Tadler 1996; Tadler 1996 citing H. Enghoff pers. comm.).

In the Spirostreptida, there is no preinsemination phase; gonopods are inserted once at the onset of copulation and remain within the vulvae for its duration. In this study, processes of ejaculate transfer are quantified for two Spirostreptida species,

TABLE 4.1 Morphometrics of *P. dipodontus* and *A. uncinatus*.

species	male	female
<i>Alloporus uncinatus</i>	mass: 8.5 ± 1.2 g; n = 116 length: 120.0 ± 12.5 mm; n = 27 width: 101.5 ± 7.3 mm; n = 27	mass: 9.2 ± 1.2 g; n = 75 length: 119.9 ± 8.5 mm; n = 11 width: 92.1 ± 4.9 mm; n = 11
<i>Poratophilus dipodontus</i>	mass: 2.2 ± 0.4 g; n = 284 length: 63.2 ± 5.1 mm; n = 89 width: 6.3 ± 0.4 mm; n = 89	mass: 5.5 ± 1.3 g; n=217 length: 78.4 ± 10.0 mm; n =50 width 9.2 ± 0.8 mm; n = 50

Alloporus uncinatus (Spirostreptidae) and *Poratophilus dipodontus* (Harpagophoridae). By separating copula pairs at varying time intervals from the onset of copulation, it is shown that sperm transfer occurs at the beginning of copulation and the proportion of ejaculate at the bottom of the vulvae increases with time. The adaptive significance of prolonged copulation, and the implications of the timing of insemination on processes of competition in Spirostreptida millipedes are discussed.

4.2 Materials and methods

4.2.1 Study Animals

Poratophilus (philoporatia) dipodontus (Harpagophoridae) and *Alloporus uncinatus* (Spirostreptidae) were collected from Moreletta, Pretoria and Mazowe, Zimbabwe respectively (see Appendix B), shortly after emergence (see Table 4.1 for morphometrics). Following collection they were separated by sex and housed in glass aquaria (50 x 30 x 50 cm) at 25°C and $\pm 75\%$ RH for at least two weeks prior to the commencement of experiments. Fresh food in the form of vegetables was provided *ad libitum*.

4.2.2 Ejaculate labelling

Tritiated [methyl- ^3H]thymidine (85 Ci/mmol, Amersham, UK) was used to label the ejaculates of males and dpm (disintegrations per min.) counted as a measure of ejaculate volume. This method was favoured above direct sperm counts (e.g. Lorch *et al.* 1993) because even though females were isolated from males in the laboratory, the

mating histories of these long-lived animals were not known, and thus the presence of sperm from previous matings could not be excluded. The radiolabelling method made it possible to distinguish the ejaculate of the labelled male.

Experimental males were injected with 50 μ l [methyl-³H]thymidine on their ventral surfaces between body segments 10 and 11. Injections took place 24h before experimental mating sequences and affected neither male copulatory behaviour nor copulation durations (Mann-Whitney U test copulation duration uninjected vs. injected males: *A. uncinatus* $U = 951$, $P = 0.139$, $n = 29$, 29 ; *P. diplodontus* $U = 815$, $P = 0.786$, $n = 28$, 28). It was assumed that ejaculates were labelled homogeneously with the isotope and evenly distributed with sperm. It is not clear whether the isotope is suspended in the ejaculate or binds to the outside of the sperm themselves. If the latter is true, then isotope distribution patterns will be linked to sperm distribution patterns. These have not yet been established. It is unlikely that the radioisotope is more dilute in larger males such that their sperm are less intensely labelled, due to the large volumes of isotope injected into these animals.

Copula pairs were generated by introducing five animals of each sex into mating arenas (glass aquaria 50 x 30 x 50 cm). At the onset of copulation, pairs were isolated in plastic jars (13 cm diameter). Copulations were either terminated at varying time intervals (*P. diplodontus* 0 - 35 mins, *A. uncinatus* 0 - 120 minutes), or allowed to terminate naturally. Copula pairs were separated by carefully uncoiling the male from the female and disengaging the gonopods.

Immediately following the separation of copula pairs, females were placed in ethyl acetate killing jars and their vulvae dissected out. Vulvae were either left intact or divided into top and bottom components (see Fig. 4.1). In *A. uncinatus* the spermatheca was in the bottom component, in *P. diplodontus* it was in the top. Vulvae were placed in 7 ml scintillation vials and vortexed for 1 min. with 0.1 ml concentrated HCl to actuate rapid tissue homogenisation. The acid was neutralised with 0.1 ml 5M NaOH prior to the addition of 3.5 ml scintillation fluid (Scintillator 299, Packard). Dpm were determined in a Packard 1600 scintillation counter (low count reject = 0; dpm multiplier = 1) and the mean dpm (± 1 s.e.) for three 10 minute determinations per sample was recorded. The tritium counting protocol automatically subtracted the standard (background count) from all measures of dpm (background count: 50.6 ± 1.95 dpm, $n = 3$).

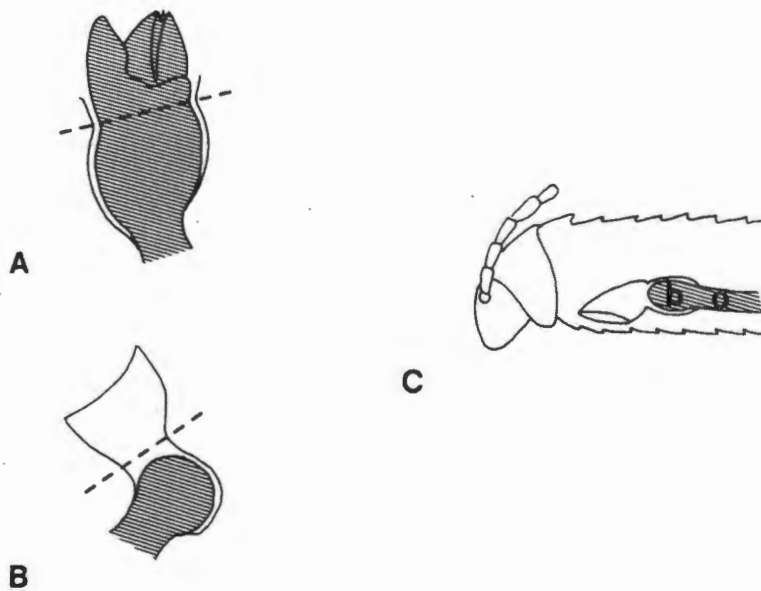


FIGURE 4.1 Diagrammatic representation of *P. diploodontus* (A) and *A. uncinatus* (B) vulvae, illustrating the point at which vulvae were divided into top and bottom components and the orientation of the vulvae within the body of the female (C). **b** = bursa; **o** = oviduct.

Vulvae from females that were mated with males injected with 50 μ l sterile, distilled water were used as controls. The range of dpm values obtained was consistent with values for background counts (dpm with background subtracted: *P. diploodontus* 5.1 - 44.1 dpm, $n = 4$, *A. uncinatus* 1.9 - 9.5 dpm, $n = 4$).

4.2.3 Statistical analyses

Statistical analyses for this and the following chapters were performed using Minitab version 7.2 (Minitab, Inc. 1989), Excel version 5.0 (Microsoft corporation 1993) and Systat for Windows (Systat, Inc. 1992) software and Zar (1984). Dpm counts did not conform to the assumptions of parametric tests and samples were too small for the data to be easily transformed to normality, thus non-parametric statistical analyses were used (Siegel and Castellan 1988). Significance tests were two-tailed except for multiple comparisons between treatments (z statistic, Siegel and Castellan 1988) for the Kruskal-Wallis one-way analysis of variance which were one-tailed. The alpha level of acceptance for all tests was 5%. Two-tailed Pearson's correlation coefficients were used in analyses of covariation involving body mass, copulation duration and dpm.

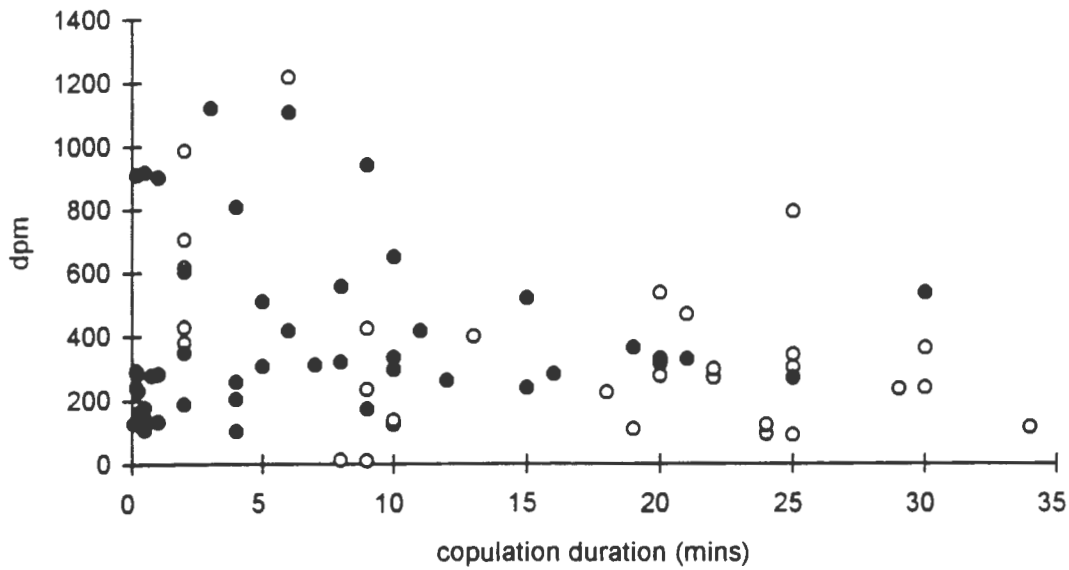


FIGURE 4.2 *P. diploodontus* total dpm (disintegrations per minute) vs copulation duration (mins). Regression equation (not shown): $dpm = 495.9 - 8.51 \text{ mins}$, $r = 0.07$, $n = 55$, $P < 0.05$.

○ = naturally terminating copulations; ● = induced copulation terminations.

4.3 Results

The genitalia remained engaged throughout the mating period (mean copulation duration *A. uncinatus* = $97.9 \pm \text{s.d. } 39.4$ mins; $n = 65$; *P. diploodontus* = $23.7 \pm \text{s.d. } 10.3$ mins, $n = 250$). Dpm counts indicate that insemination occurs shortly after the onset of copulation and is almost instantaneous in some animals. The high dpm counts over the first phase of copulation indicate that the volume of ejaculate that remains within the vulvae following copulation is less than the total volume a male transfers (Figs 4.2, 4.3). In both species, dpm values for animals with naturally occurring (N) and induced (I) terminations were similar, indicating that the experimental protocol did not interfere with normal processes of ejaculate transfer (Mann-Whitney U tests: *P. diploodontus* N vs I; $U = 1211$; $n = 33, 45$; $P > 0.1$; *A. uncinatus* N vs I; $U = 233$; $n = 12, 22$; $P > 0.1$).

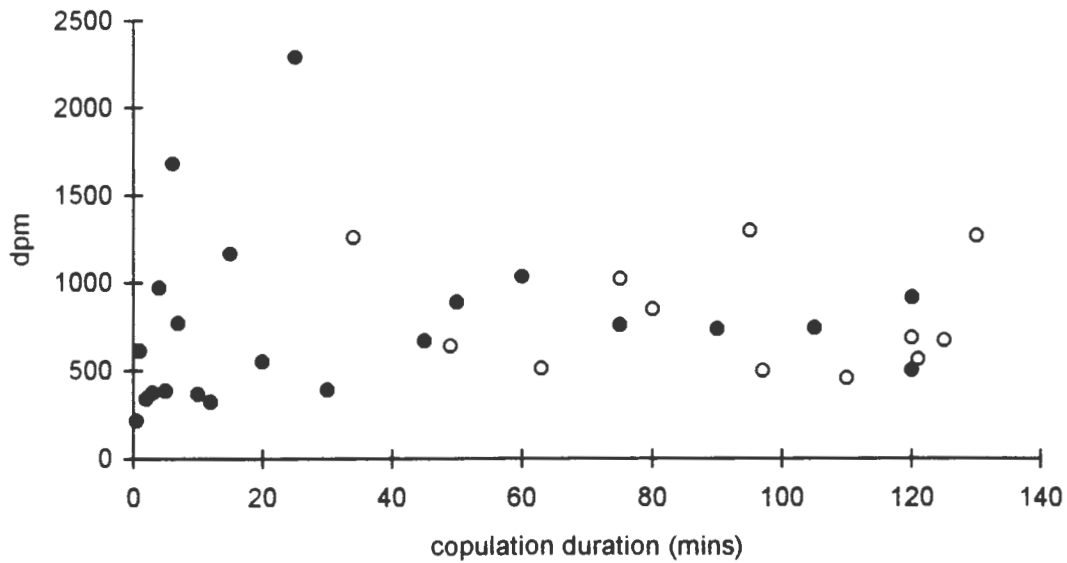


FIGURE 4.3 A. *uncinatus* total dpm (disintegrations per minute) vs copulation duration (mins). Regression equation: $dpm = 753.28 + 0.39 \text{ mins}$, $r = 0.002$, $n = 34$, $P > 0.05$.

○ = naturally terminating copulations; ● = induced copulation terminations.

Substantial variation in dpm was apparent over all copulation durations. This may be the consequence of intraspecific variation in ejaculate volume transferred (e.g. Lewis and Austad 1990, see Chapter 1) or selective storage of ejaculates by females (see Chapter 3). There was no relationship between copulation duration and either male or female body mass for either species (*P. diplodontus* copulation duration (mins) vs female body mass (g): $r = 0.003$, $n = 137$, $P > 0.1$, copulation duration (mins) vs male body mass (g): $r = 0.158$, $n = 137$, $P > 0.05$; *A. uncinatus* copulation duration (mins) vs female body mass (g): $r = 0.067$, $n = 35$, $P > 0.1$, copulation duration (mins) vs male body mass (g): $r = 0.122$, $n = 35$, $P > 0.1$).

In both *P. diplodontus* and *A. uncinatus* the proportion of dpm placed at the bottom of the vulvae increases shortly after the onset of copulation (Figs 4.4, 4.5). This increase occurs over the same period as the drop in total dpm suggesting that the proportional increase in dpm at the bottom may simply be the consequence of ejaculate loss from the top. In *P. diplodontus*, there is no significant relationship between total dpm placed at the bottom of the vulvae and copulation duration when all data points are included in the analysis. When the two high values (>500) are excluded, a strong relationship is

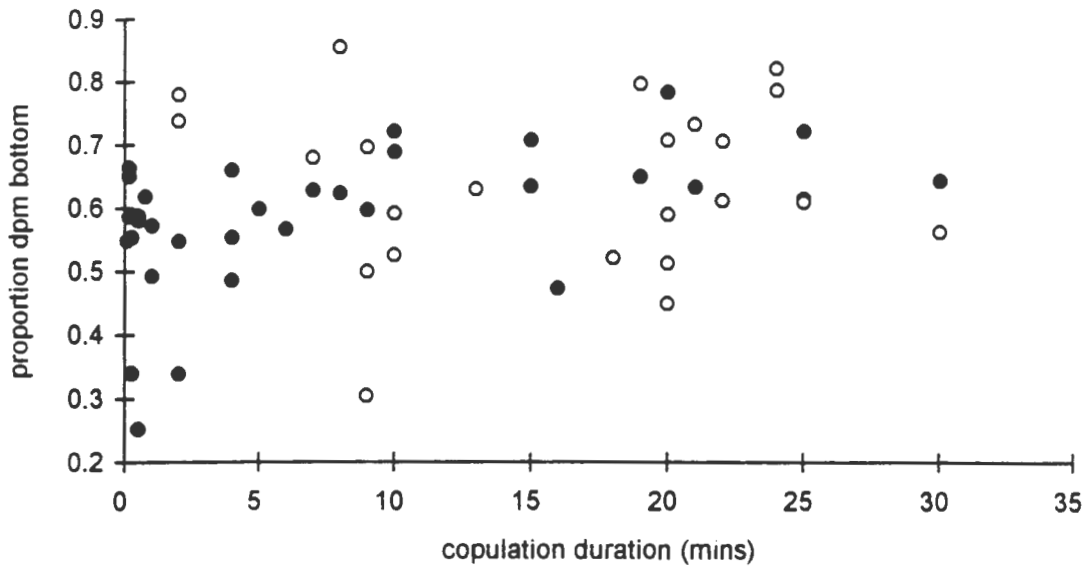


FIGURE 4.4 *P. dipodontus* proportion dpm (disintegrations per minute) at the bottom of the vulvae vs copulation duration (mins). Regression equation: $\text{proportion dpm bottom} = 0.55 + 0.004 \text{ mins}$, $r = 0.1$, $n = 56$, $P < 0.02$.

○ = naturally terminating copulations; ● = induced copulation terminations

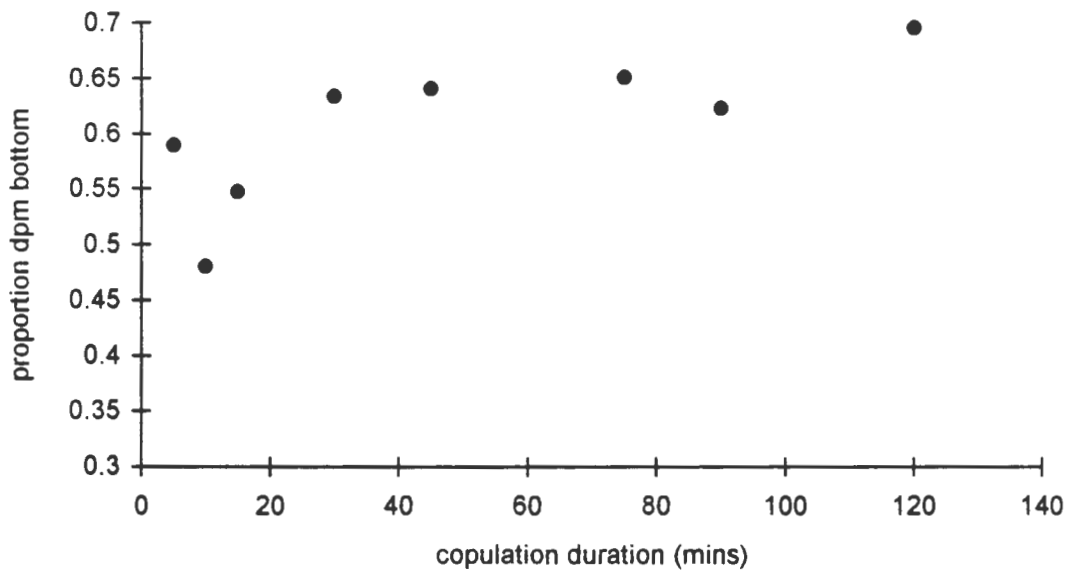


FIGURE 4.5 *A. uncinatus* proportion dpm (disintegrations per minute) at the bottom of the vulvae vs copulation duration (mins). Regression equation: $\text{proportion dpm bottom} = 0.54 + 0.001 \text{ mins}$, $r = 0.58$, $n = 8$, $P < 0.05$.

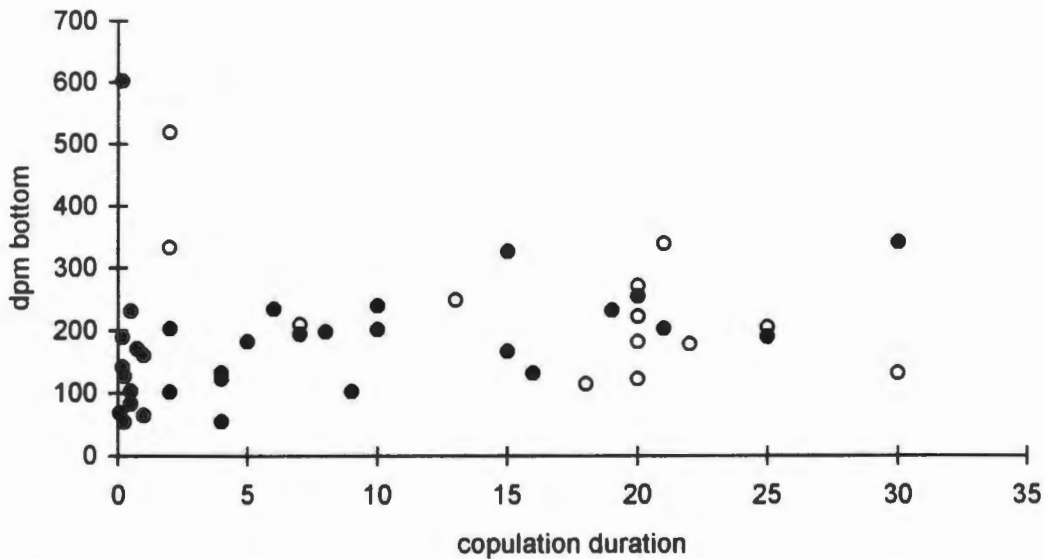


FIGURE 4.6 *P. dipodontus* total dpm (disintegrations per minute) at the bottom of the vulvae vs copulation duration (mins). All data points included: Regression equation: $\text{dpm bottom} = 7.93 + 0.01 \text{ mins}$; $r = 0.13$, $n = 45$, $P = 0.395$; outliers omitted: Regression equation: $\text{dpm bottom} = 0.95 + 0.05 \text{ mins}$; $r = 0.18$, $n = 43$, $P < 0.01$.
 ○ = naturally terminating copulations; ● = induced copulation terminations.

apparent (Fig. 4.6), suggesting that the shift in proportion of ejaculate placed at the bottom is not simply the consequence of loss of ejaculates in close proximity to the gonopore. (A similar analysis was not possible in *A. uncinatus* due to the small sample size).

4.4 Discussion

In both *P. dipodontus* and *A. uncinatus*, insemination occurred instantaneously at the onset of copulation and in both species a smaller volume of ejaculate remained in the vulvae following copulation than was transferred, owing to ejaculate loss shortly after the onset of copulation. Preliminary data from other Diplopod species (*Chersastus spp.* (Spirobolida), M. Cooper pers. comm.; *Calostreptus sp.* (Spirostreptida), see Chapter 7), support this finding and suggest this to be a common or possibly universal Juliform trait (see Appendix A).

The phenomenon of males transferring greater volumes of ejaculates than remain in the spermathecae following copulation is common in other taxa (Lefevre and Jonsson 1962; Sivinski 1980; Gromko *et al.* 1984; Brillard and Bakst 1991; Otronen and Siva-

Jothy 1991; Eady 1994b; Eberhard 1996). In *A. uncinatus* and *P. diplodontus*, several non-exclusive processes may account for this loss:

a. Self sperm displacement

Mechanisms of ejaculate competition and the timing of insemination can be interrelated. For example, where male Odonata compete via displacement, insemination typically takes place after the manipulation of rival ejaculates (Miller and Miller 1981; Fincke 1984; Waage 1986a, Cordero and Miller 1992), enabling males to manipulate rival sperm without compromising their own sperm. The same is true of the bushcricket *Metaplastes ornatus* (von Helversen and von Helversen 1991). Early insemination (and a lack of stratification) implies that males would be unable to selectively remove rival ejaculates without also affecting their own, and in *P. diplodontus* and *A. uncinatus*, the loss of ejaculates shortly after the onset of copulation may be a consequence of unavoidable self sperm removal (G. Parker personal communication in Birkhead and Hunter 1990; Eady 1994b).

b. Competition through ejaculate flushing

Similarly, ejaculate loss may also be the consequence of an attempt to flush out rival sperm from the vulvae. Previous studies have shown that where males preempt previous ejaculates by flushing them from the stores, a greater volume of sperm is transferred than remains following copulation (e.g. Eady 1995).

c. Passive loss as a consequence of genital movements

Finally, bursal muscle contractions and/or cyclic telopodite retraction-release cycles may cause ejaculates in close proximity to the gonopore to be lost, and males may transfer large ejaculates to compensate for this.

Spirostreptida females have been observed to groom their genitalia following copulation and the excess sperm may form a nuptial gift (Thornhill 1974; Gwynne 1984). (It is equally possible that grooming has no adaptive significance and that females are simply cleaning themselves).

Detectable redistribution of ejaculates from the gonopore to the bottom of the bursae happens at the same time as ejaculate loss. In *A. uncinatus*, the stores are in the bottom component and redistribution may be adaptive in terms of fertilisation success. However, this redistribution also occurs in *P. diplodontus* where the stores are in the top component. It is therefore suggested that the movement of ejaculates to the bottom is simply a consequence of movement of ejaculates away from the gonopore and into the vulval sacs. Because sperm are non-motile, this movement may be either passive, male-mediated via the telopodites or female-mediated via vulval muscle contractions.

In *A. uncinatus*, the duration of copulation is male-mediated and increases with increasing male bias in the OSR (Telford and Dangerfield 1990a). Consequently, prolonged copulation has been interpreted as a form of mate guarding. This interpretation is consistent with the early onset of insemination and the absence of a relationship between copulation duration and ejaculate volume transferred in both *A. uncinatus* and *P. diplodontus*. However, it is necessary to consider the adaptive significance of maintaining genital contact throughout copulation. Postcopulatory passive phases and non-contact guarding are both mechanisms by which males guard their mates without maintaining genital contact (Parker 1970a; Thornhill and Alcock 1983; Alcock 1994), thereby extending mating time, but not the duration of copulation. This suggests that the maintenance of genital contact is not a prerequisite for guarding, and that the engagement of genitalia may have a functional significance of its own.

Genital contact has also been shown to be important in the induction of female non-receptivity (Eberhard 1996). This hypothesis remains to be tested in the Spirostreptida¹.

In *A. uncinatus* and *P. diplodontus*, prolonged genital contact may facilitate ejaculate entry from the anterior bursae into the spermathecal ampullae (micro-redistribution, not detectable using this methodology):

a. Males may directly move ejaculates into the stores. Prolonged genital contact has previously been associated with "strategic ejaculate positioning" (Alcock 1994). In *Mnais pruinosa pruinosa*, males are believed to reposition gametes to improve their fertilisation success (Siva-Jothy and Tsubaki 1989) and in *Chelymormpha alternans*

¹ A positive relationship was not evident in preliminary data looking at the relationship between copulation duration and the refractory period between matings.

(Rodríguez 1994), *Bombyx mori* (Suzuki *et al.* 1996) and *Callobruchus maculatus* (Eady 1994b), a refractory period that is associated with ejaculate movement from the site of insemination to the stores occurs after insemination. Direct movement of ejaculates into the spermathecae would only be possible where the telopodites have direct access to the spermathecae. In *P. diplodontus* the telopodites may have access to the spermathecal openings. It is unlikely that they enter the ampullae due to the broad configuration of the telopodite tip. In *A. uncinatus*, although the distal telopodites reach the bursae, they do not enter them (see Chapter 3). Consequently, processes of ejaculate movement into the spermathecae are probably either passive or female-mediated and do not explain the prolonged duration of copulation.

b. Alternatively, males may induce females to store ejaculates through copulatory courtship (Eberhard 1985). As mentioned in Chapter 3, the bursal muscles that attach to the bursae in Spirostreptida vulvae may sanction syncopulatory female choice. Morphological evidence for a female-mediated process of sperm redistribution may be found in the bursal muscles that fan out from the spermathecae to the bursal walls. If muscles are regulated by females to control sperm access, males may be selected to stimulate females to store their ejaculates. Cyclic telopodite movements, and the interaction of the distal telopodites with the anterior bursae may function to effect this stimulation.

In summary, early insemination is consistent with the hypothesis that prolonging the duration of copulation is a form of mate guarding. However, the possibility that males stimulate females to store sperm illustrates that mate guarding itself may not totally account for the evolution of prolonged copulation. Improved fertilisation success may compensate for the suggested trade-off between time invested in copulatory mate guarding and future mating opportunities (Parker 1970a; Thornhill and Alcock 1983; Alcock 1994).

CHAPTER FIVE

SPERM REMOVAL IN *PORATOPHILUS DIPLODONTUS* (HARPAGOPHORIDAE)

5.1 Introduction

Morphological characters of both male and female genitalia suggest direct ejaculate manipulation in the Harpagophoridae; bursae are situated in close proximity to the gonopore and the distal tip of the telopodite bears spines and plates similar to those previously implicated in ejaculate displacement in Odonata (e.g. Waage 1984, 1986a; see Chapter 1) and the beetle *Tenebrio molitor* (Gage 1992).

In this chapter, radioactive labelling techniques are used to examine mechanisms of competition in *P. dipodontus*, and patterns of ejaculate transfer and subsequent storage are interpreted in the context of genital functional morphology. Using a simple genital manipulation, the following hypotheses are tested: (a) that the distal end of the telopodite displaces previous ejaculates and (b) that the distal end of the telopodite functions in ejaculate placement.

This is not the first study to attempt an empirical demonstration of genital function through a manipulation. For example, Sengun (1944 cited Shapiro and Porter 1989) manipulated the genitalia of *Bombyx mori* to show the absence of a lock and key mechanism. Ono *et al.* (1989) blocked the penis of the tree cricket *Trujalia hibinonis* to demonstrate that sperm removal was a product of ejaculate flushing, and Rodriguez *et al.* (submitted, cited Eberhard 1996) manipulated the length of the genital flagellum of the beetle *Chelymorpha alternans* to illustrate its significance in female choice. This is, however, the first study to couple an empirical verification of genital function with a quantification of its effect on direct sperm removal. It is suggested that this approach

could be usefully applied in a variety of taxa where sperm removal appears to be performed exclusively by the male genitalia.

5.2 Methods

Animals were collected, housed and maintained as in Chapter 4.

5.2.1 Ejaculate labelling

Mechanisms of sperm competition were elucidated by labelling the ejaculates of males with tritiated thymidine ([methyl-³H]thymidine; 85 Ci/mmol, Amersham, UK) and examining (a) the relative ejaculate volumes that successive mates contribute to the sperm stores and (b) their distribution within the stores.

5.2.2 Basic mating sequences

To determine if sperm displacement occurred, serial matings with males having unlabelled sperm and males whose sperm had been labelled with tritiated thymidine were conducted. Males used in these mating sequences were labelled with the isotope 24 hours before the start of the experiment. 'Labelled' males (L) were injected on their ventral surfaces between body segments 10 and 11 with 50 µl [methyl-³H]thymidine, using a 50 µl Hamilton syringe. To control for possible side-effects of the injection, 'unlabelled' males (UL) were injected with an equivalent volume of sterile, distilled water. It was assumed that ejaculates were homogeneously labelled with the isotope (see Chapter 4).

Two experimental series, each comprising two double mating sequences were conducted (Table 5.1). In the first sequence each female was mated first with a labelled male and then with an unlabelled male (L-UL). These results were used to quantify the ejaculate contribution of the first male. In the second sequence, the order was reversed and each female was mated first with an unlabelled and then with a labelled male (UL-L), thus quantifying the contribution of the second male. In the first series, matings followed each other immediately and in the second, the delay between matings was 24 hours.

TABLE 5.1 *P. dipodontus* mating sequences. UL = unlabelled male; L = labelled males.

(M) following either of these indicates that a telopodite manipulation was performed on that male's gonopods.

<i>mating sequence</i>	<i>purpose</i>
UL only	control
L only	ejaculate contribution of male 1 - single mating
L - UL (0 & 24 hour)	ejaculate contribution of male 1 - double mating
UL - L (0 & 24 hour)	ejaculate contribution of male 2 - double mating
L - UL (M)	role of telopodite in displacement
L (M)	role of telopodite in placement or self sperm displacement

Females were also mated once with labelled males (L) to provide information about the contribution of the first male to mate with a female when this was not followed by a second mating, and dpm of vulvae from females who had mated once with unlabelled (UL) males were determined as a control.

5.2.3 Telopodite manipulation sequences

The functional morphology of the gonopods of *P. dipodontus* reveals a possible mechanism of ejaculate manipulation: at its distal end, the telopodite bears several lamellae including a comb with several teeth that rotate about its axis when the telopodite is retracted and released (see Figs 2.5; 2.32). To test the role of the telopodite in ejaculate displacement, a second series of experiments in which the gonopods were manipulated was conducted. Because gonopods are chitinous, non-inflatable and paired, one side can be manipulated while the other functions as a control. To economise on the use of the radioisotope (and the number of males that had to be sacrificed), labelled males from the basic mating sequence experiments (above) were reused for this experiment. Because these males were mating for a second time, and either 10 days (L-UL(M) males) or 21 days (L(M) males) following labelling, direct comparisons between these data and those from the basic mating sequences are not made.

The manipulation was performed as follows: the gonopods of manipulated males were everted with forceps and the telopodite arm on the left gonopod was severed at its point of emergence from the coxite (Fig. 5.1). This manipulation did not appear to affect the behaviour of the males, who walked, mounted females etc. as normal.

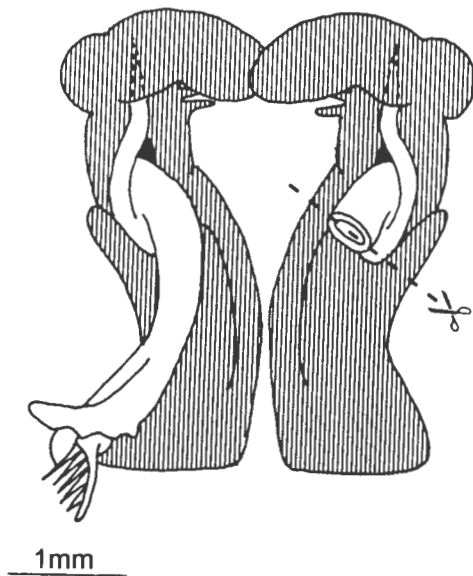


FIGURE 5.1 *P. dipodontus* gonopods illustrating point at which the left telopodite was severed.

□ = telopodite; ▨ = coxite.

Telopodite manipulations were performed 24 hours before the inclusion of these males in any experiments.

Two mating sequences involving manipulated males were performed:

- a. To determine the function of the telopodite arm in the displacement of previous ejaculates, the left and right vulvae of females that had mated first with a labelled male and then with an unlabelled manipulated male were compared (L-UL(M)).
- b. To determine the function of the telopodite arm in ejaculate placement, the total dpm and the proportion dpm placed at bottom of the left and right vulvae of females that had mated once with a manipulated labelled male (L(M)) were compared.

5.2.4 Generation of copula pairs

Copula pairs were generated by placing up to five animals of each sex in glass aquaria similar to those in which they were housed. Upon their formation, pairs were translocated from the aquaria to plastic beakers (13 cm diameter) and the duration of each mating was recorded. Both animals appeared to be unaffected by the move which was necessary to prevent interference from single males (pers. obs. and see Telford and Dangerfield 1993a and b).

5.2.5 Scintillation counting

Immediately following the termination of their second mating, females were placed in ethyl-acetate killing jars. Their vulvae were dissected out and each vulva was divided into top and bottom components as in Chapter 4. These were placed separately into 4 ml pony vials and vortexed with 0.1 ml concentrated HCl to disintegrate the tissue (and prevent differential quenching). The acid was neutralised with 0.1 ml 5M NaOH prior to the addition of 3.5 ml scintillation cocktail (Scintillator 299, Packard). Dpm were determined in a Packard 1600 scintillation counter (low count reject = 0, dpm multiplier = 1) and the mean dpm (± 1 s.e.) for three 10 minute determinations per vial was recorded. The tritium counting protocol automatically subtracted the background count (50.6 ± 1.9 dpm, $n = 3$) from all measures of dpm.

Dpm values of vulvae from females who had mated once with unlabelled (UL) males were consistent with values from background counts (Dpm of control females with background subtracted: 5.1- 12.8 dpm).

5.3 Results

5.3.1 Interpreting relative ejaculate contribution: V_2

Because ejaculate volumes do not necessarily correspond to paternity (see Chapter 1), the relationship between successive males' ejaculates cannot be explained in terms of P_2 (the proportion of offspring sired by the last male to mate). Consequently, a term that describes relative ejaculate volume rather than relative paternity, is proposed. V_2 is the proportion of ejaculate contributed to the sperm stores of the female by the second male to mate with her in a double mating sequence.

$$V_2 = \text{sperm volume male 1} / (\text{sperm volume male 1} + \text{sperm volume male 2})$$

Importantly, females were not reared in laboratory cultures due to time constraints and difficulties associated with inducing oviposition under laboratory conditions. Animals were collected close to the onset of mating activity but it was not possible to distinguish virgins from non-virgins. Because prior sperm cannot be accounted for, this study yields a measure of $V_{(x)}$ and $V_{(x+1)}$.

$$V_{(x-1)} = \text{sperm volume male } (x+1) / (\text{sperm volume male } x + \text{sperm volume male } (x+1))$$

In this chapter I shall refer to $V_{(x)}$ and $V_{(x+1)}$ as V_1 and V_2 respectively.

5.3.2 Basic mating sequences

Because data are not normally distributed, non-parametric statistics were used for comparisons (see previous chapter for details of statistical analysis procedures). A difference in the time delay between matings had no effect on the resultant dpm of the vulvae for either of the double mating sequences (L-UL 0 vs 24 hr Wilcoxon test: $T = 73$, $n = 9,10$; $P = 0.289$; UL-L 0 vs 24 hr Wilcoxon test: $T = 83$, $n = 9,10$, $P = 0.859$), and the data from the 0 hr and 24 hr delay series were combined.

There were significant differences between the total dpm values of the different mating sequences (Kruskal Wallis test: L vs L-UL vs UL-L; $H = 21.85$, $df = 2$, $P = 0.0$; Fig. 5.2). Single matings with labelled males yielded significantly higher dpm counts than either of the double mating sequences (Mann-Whitney U test: L only vs UL-L: $U = 754$, $n = 27, 19$, $P = 0.0021$; L only vs L-UL: $U = 801$, $n = 27, 19$, $P = 0.0$). A comparison of the L-UL and UL-L sequences indicated that dpm counts were higher when labelled males copulated second (Mann-Whitney U test: L-UL vs UL-L: $U = 404$, $n = 19, 19$, $P = 0.026$).

Data were log transformed to yield a symmetric distribution and enable the calculation of geometric means (L: mean dpm = 263.03, $n = 27$; L-UL: mean dpm = 93.32, $n = 19$; UL-L: mean dpm = 154.88, $n = 19$). These were used to estimate V_2 and the proportion of ejaculate removed by second males in the basic sequences.

Second males have a V_2 of 0.62. To achieve this value, 64.52% of the previous ejaculate must be removed from the vulvae ($1 - (\text{dpm L-UL})/(\text{dpm L})$). Because prior sperm are not accounted for and females may not have been virgins, this may be a conservative estimate of total removal.

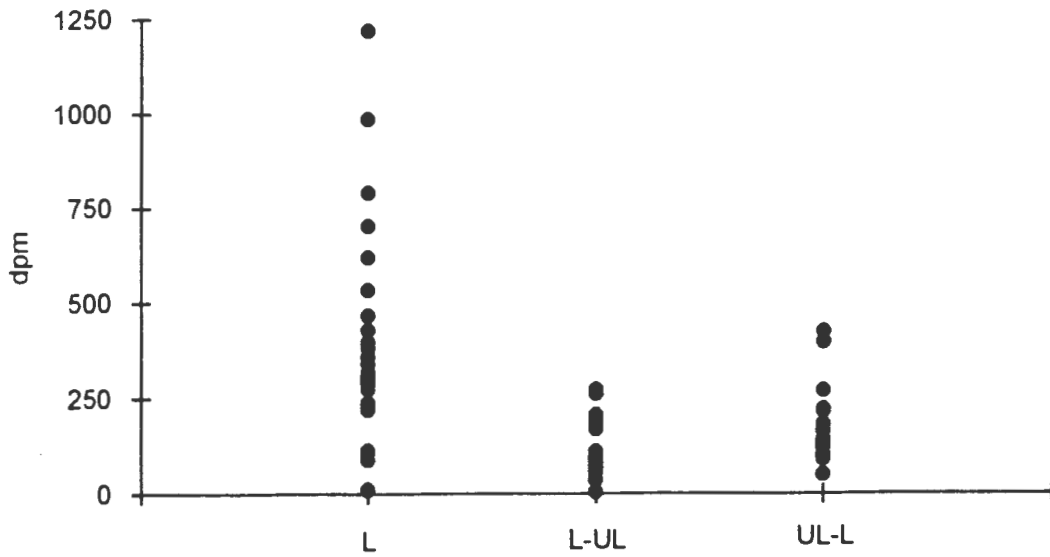


FIGURE 5.2 Dpm counts from *P. diplo dontus* mating sequences. Each point represents the count from a single female. L: $n = 27$; L-UL: $n = 19$; UL-L: $n = 19$.

TABLE 5.2 Proportion dpm at bottom of *P. diplo dontus* vulvae.

<i>mating sequence</i>	<i>proportion dpm at bottom</i>	\pm s.d.	<i>n</i>
L	0.65	0.11	17
UL - L	0.59	0.16	10
L - UL	0.56	0.20	6

The proportion dpm placed at the bottom of the vulvae in each mating sequence is presented in Table 5.2. The data from each mating sequence were arcsin transformed and compared, and showed no differences in the proportion dpm placed in the bottom vulval component (Kruskal-Wallis Test: $H = 3.58$, $df = 2$, $P = 0.168$, Mann-Whitney U tests: L vs UL-L: $U = 254$, $n = 17, 10$, $P = 0.436$; L vs L-UL: $U = 215.5$, $n = 17, 6$; $P = 0.441$; UL-L vs L-UL: $U = 88$, $n = 10, 6$, $P = 0.786$). Because there were no differences, it is concluded that ejaculates are not stratified between the top and the bottom of the vulval sacs. This measure excludes a fine examination of the contents of the vulvae and the spermatheca and cannot exclude microlayering.

5.3.3 Manipulation sequences

Data from the manipulation sequences were normally distributed but with unequal variances. Consequently non-parametric statistics are used for comparisons while the untransformed data are used for the calculation of means.

As mentioned, a direct comparison of dpm values between the manipulated and unmanipulated (basic sequences) data sets is not made due to differences in the delay between labelling and mating in the manipulated sequences (1 day unmanipulated males, 10 days for L-UL(M) males, 21 days for L(M) males), and the use of twice-mated males in the manipulation sequences.

Assumptions

The comparison of the dpm of left and right vulvae in the manipulation sequences makes the assumption that when unmanipulated males copulate with females, there are no differences between the left and right vulvae in either total dpm or the proportion of dpm held at the bottom of the vulvae. This was tested by comparing the contents of those vulvae used in the L, UL-L and L-UL mating sequences.

No left-right differences occurred in any of the mating sequences (Wilcoxon tests: L only left vs right: $T = 69.5$, $P = 0.5$, $n = 19$; UL-L left vs right: $T = 130$, $P = 0.165$, $n = 19$; L-UL left vs right: $T = 69.5$, $P = 0.5$, $n = 27$) or in the proportion of ejaculate placed at the bottom of the vulvae by unmanipulated males (L only left vs right: Wilcoxon test: $T = 88$, $P = 0.603$, $n = 17$). It is concluded that the above assumptions are valid.

The results of the manipulation mating sequences are presented in Table 5.3.

L(M) sequence:

The dpm values for L(M) mating sequence give an indication of the contribution of first male to the stores prior to the second mating. The results of the L(M) matings indicate no difference in the total dpm content of the left and right vulvae (Wilcoxon test L(M) left vs right: $T = 92$, $P = 0.478$, $n = 13$). Because there are no differences in

TABLE 5.3 Results of mating sequences involving manipulated males

<i>mating sequence</i>	<i>vulval side</i>	<i>mean dpm</i>	\pm <i>s.d.</i>	<i>n</i>
L - UL (M)	left	111.3	69.1	19
	right*	151.3	97	19
L (M)	left	265.2	161.7	17
	right*	274	151.3	17
		mean proportion dpm bottom		
L(M)	left	0.657	0.121	13
	right*	0.709	0.135	13

* = side penetrated by gonopod without telopodite

dpm between left and right vulvae in the L(M) mating sequence, it can be concluded that the telopodite does not play a role in either ejaculate placement or self sperm displacement.

No differences in the proportion dpm placed in the bottom vulval component indicate that the telopodite is also not implicated in ejaculate movement to the bottom of the vulvae (Wilcoxon test $T=63$, $P=0.235$, $n=17$).

The canal that runs along the telopodite has previously been termed the "sperm canal" due to speculation about its role in ejaculate transfer (ejaculates and products of the coxal gland were thought to be pumped from the base of the coxite into the vulvae via the canal, Attems 1928; Demange 1959). Because ejaculate transfer is not affected by telopodite removal, it can be concluded that the telopodite canal does not play a role in ejaculate transfer.

L-UL(M) sequence:

A comparison of the contents of the two sides of vulvae from the L-UL(M) mating sequence indicated a significantly lower dpm count for the left vulva, which was the vulva associated with the intact telopodite arm (Wilcoxon test: L - UL(M) left vs right: $T=170$, $P=0.003$, $n=19$). The difference in dpm between the left and right vulvae can be attributed to telopodite function and it can be concluded that the telopodite removes 26.46% of the previous male's ejaculate ($1 - (\text{dpm left})/(\text{dpm right})$).

There is a significant difference between this value and that from the basic mating sequences ($\chi^2 = 94.61$, $df = 1$, $P > 0.05$). The telopodite is therefore only partially responsible for ejaculate removal.

5.3.4 Copulation duration

The copulation durations of first and second males in the basic mating sequences did not differ (mean copulation duration male 1: 26.3 ± 7.59 min., $n = 38$; mean copulation duration male 2: 27.2 ± 8.1 min., $n = 38$; Wilcoxon test: $T = 406.5$, $P = 0.607$, $n = 38$). However, copulations with manipulated males were significantly shorter than those with unmanipulated males (mean copulation duration unmanipulated 24.2 mins ± 9.55 , $n = 112$, mean copulation duration manipulated 16.7 mins ± 7.13 , $n = 35$; Mann-Whitney U test unmanipulated vs manipulated: $U = 1306$, $n = 35, 93$, $P = 0$). This result indicates that telopodite function and copulation duration may be related.

5.3.5 Covariation in dpm.

Ejaculate volume has previously been related to male body mass with larger males transferring larger ejaculate volumes (Rubenstein 1989; Berrigan and Locke 1991). The absence of a relationship between male body mass and dpm (disintegrations per minute) indicated no relationship between male body mass and ejaculate volume transferred and also no difference in the intensity of radioisotope labelling of ejaculates of males of different sizes (Pearson's correlation coefficient: UL-L: $r = 0.31$, $P = 0.179$, $n = 19$, L only $r = 0.32$, $P = 0.1$, $n = 27$, both two-tailed test). If, however, larger males transferred larger ejaculates *and* were less intensely labelled, a relationship between ejaculate size and body mass would be obscured. Sperm displacement was also not related to copulation duration (L-UL dpm vs copulation duration male 2: $r = 0.17$, $P = 0.461$, $n = 19$). If vulval size was positively correlated with female body mass and this size difference was related to vulval holding capacity, a relationship between female body mass and dpm would be expected. No such relationship was evident (L only: $r = 0$, $P = 0.93$, $n = 27$; UL-L: $r = 0.41$, $P = 0.08$, $n = 19$).

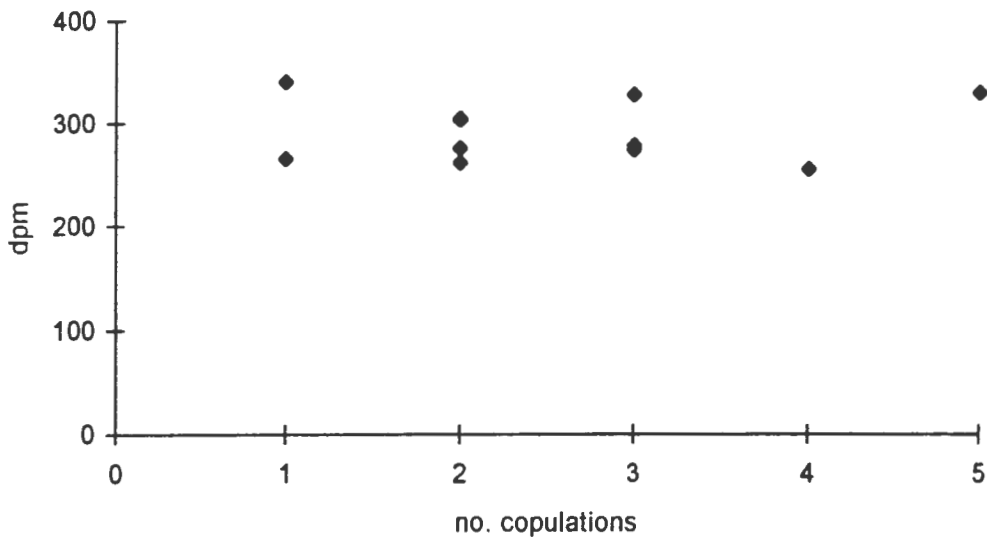


FIGURE 5.3 Preliminary investigation into *P. dipodontus* vulval capacity. Dpm (disintegrations per minute) is plotted against copulation number. ($r = 0$; $P = 0.94$; $n = 12$)

5.4 Discussion

Single matings yielded higher dpm counts than those for either of the double mating sequences, suggesting that previous ejaculates may limit the storage of later ones. The notion that vulval capacity is limited is supported by preliminary data: with up to five successive matings with labelled males, there is no increase in total vulval dpm (Fig. 5.3).

Following a double mating sequence, V_2 was 0.62, suggesting that the last male has volumetric superiority. It is difficult to interpret this value with respect to paternity assurance because patterns of (a) sperm distribution within the ejaculate and (b) ejaculate distribution within the stores are unclear. The following discussion is based on the assumption that ejaculates are homogeneously distributed with sperm. It is acknowledged that this may be an oversimplification.

If ejaculates are stratified within the spermathecal ampullae such that the sperm of the last male have easier access to the emerging ova, the last male may have greater paternity assurance than a P_2 of 0.62. For example, in the bug, *Oncopeltus fasciatus*, a 50% reduction in ejaculate resulted in 90-100% fertilisation displacement (Walker

1980). If, however, ejaculates of different males mix within the stores such that they are used for fertilisation in proportion to their numbers, $P_2 \rightarrow V_2$. Mixing of ejaculates is associated with temporal delays between insemination and oviposition; these are typical for millipedes. Selective uptake of ejaculates into the spermathecae by females is equally possible, in which case V_2 would bear no relation to P_2 .

To achieve a V_2 value of 0.62, at least 64.52% of the sperm already present in the vulvae must be removed. Due to most sperm competition studies not tracing ejaculates directly, and inferring mechanisms of competition from measures of P_2 , the volumes of ejaculates that are typically removed in other taxa are not known. Notable exceptions are the bug *Oncopeltus fasciatus* (50% removal, Walker 1980), the bushcricket *Metaplastes ornatus* ($\pm 85\%$ removal, von Helversen and von Helversen 1991) and several Odonata (damselflies: 40-50% removal in *Lestes vigilax*, Waage 1982; 88-100% in *Calopteryx maculata*, Waage 1979a; 98% removal in *Calopteryx dimidita*, Waage 1984; up to 100% removal in *Mnais pruinosa pruinosa*, depending on the duration of copulation, Siva-Jothy and Tsubaki 1989; dragonflies: 41-87% in *Sympetrum danae*, Michiels and Dhondt 1988; 57-75% in *Erythemis simplicicollis*, McVey and Smittle 1984; up to 100% in *Orthetrum cancellatum*, Siva-Jothy 1987a). In the tree cricket *Truljalia hibinonis*, the authors assume that the last male's ejaculate directly replaces that which was removed, and conclude that 87.5% of the semen from previous males is displaced (Ono *et al.* 1989). This assumption may not be valid - males may only partially replace removed ejaculates, or inseminate greater volumes than are removed.

Why do second *P. dipodontus* males only partially remove previous ejaculates?

This may be an effect of the early timing of insemination in *P. dipodontus*; males would be unable to remove rival ejaculates without also affecting their own (G. Parker personal communication in Birkhead and Hunter 1990). Additionally, they may not have access to all areas of the spermathecae. The distal telopodite tip is broader than the spermathecal ampullae and although males probably have access to the furrow and apodemic tube, direct removal from the ampullae themselves is not possible. If females are able to selectively store ejaculates by controlling ejaculate entry into the spermathecal ampullae (a proposed function of bursal muscles, see Chapter 3), later males would only be able to displace "unselected" sperm. Thus, with partial sperm removal, females may benefit from the incitement of direct sperm competition

(Birkhead and Møller 1992; Eberhard 1996). Not only do they accumulate sperm from several males through mating multiply (turning the spermatheca into a "sperm cache" *sensu* Siva-Jothy and Hooper 1995), they also ensure that only the sperm of "desirable" males remain behind to fertilise the ova.

Mechanisms of displacement

Observations of the precise role of the genitalia are rarely provided due to changes in genital size and shape during sperm transfer (for example, Odonata penises are inflated during copulation) and the operation of the genitalia within the female reproductive tract. Because we do not have recourse to direct observation in most species, empirical verification of genital function can be achieved only indirectly via either sequential disruption of copulation or direct manipulations of the genitalia themselves.

In spite of many studies associating genital morphology with sperm displacement, the role of the male genitalia in sperm removal has only been empirically demonstrated for two beetles (*Psacotheta hilaris*, Yokoi 1990 cited Eberhard 1996; *Tenebrio molitor*, Gage 1992), and the Odonata (e.g. Waage 1979a, but see below). In these studies, the components of the male genitalia responsible for displacement were isolated by disrupting mating and looking at sperm distribution. Because removal is not quantified, although they positively associate the genitalia with removal, they do not show that displacement is exclusively a product of genital function. Thus while the association of genital components with sperm may indicate a role in displacement, it does not exclude other modes of displacement that may work in conjunction with the genitalia to effect removal. Without a quantification of the role of the genitalia in processes of ejaculate displacement, the significance of female processes and other components of the male genitalia may be underestimated.

In *P. dipodontus*, gonopod manipulation experiments implicate the telopodite in ejaculate removal, but also suggest that it is not solely responsible for it. The displacement that is not accounted for by the telopodite is unlikely to be a product of the other gonopod components due to their superficial position within the vulvae (see Chapter 3). It may be a consequence of (previous) sperm ejection by females in response to male stimulation (Villavaso 1975a; Eberhard 1996). This is discussed in detail in the following chapter. Alternatively, displacement may be the result of a flushing effect caused simply by ejaculate transfer (Etman and Hooper 1979; Ono *et al.*

1989). A manipulation similar to that of Ono *et al.* (1989), in which the penis was blocked, was attempted in the Spirostreptida to quantify the flushing function. Due to ejaculates being partly composed of a secretion from the "*gland de prostatique*" (Brölemann 1917), blocking the penis was not sufficient to stop ejaculate transfer.

This study thus illustrates that while the association of genital components with sperm may indicate a role in displacement, it does not exclude other modes of displacement that may work in conjunction with the genitalia to effect removal. Without a quantification of the role of the genitalia on processes of ejaculate displacement, the significance of female processes in patterns of sperm precedence may be underestimated.

CHAPTER SIX

FEMALE MEDIATION OF SPERM COMPETITION IN *ALLOPORUS UNCINATUS* (SPIROSTREPTIDAE)

6.1 Introduction

Male adaptations to sperm competition are widely recognised and include mate guarding (e.g. Parker 1974; Waage 1979b; Sillén-Tullberg 1981; Telford and Dangerfield 1990a, b), removal of rival ejaculates (e.g. Waage 1979a) and stratification of ejaculates such that they are displaced to regions of the spermathecae where fertilisation is less likely to occur (Siva-Jothy 1988; Walker 1980). Female adaptations are less obvious and have often been considered to be of secondary importance (Knowlton and Greenwell 1984; Birkhead and Møller 1993). Females can store sperm for long periods and in the field successive matings are not always immediate. Where copulation does result in insemination, insemination does not always result in fertilisation (Eberhard 1985, 1991) and females may exercise ultimate control over offspring paternity (cryptic female choice, *sensu* Thornhill 1983). Processes of postcopulatory (but prefertilisation) female choice within the female reproductive tract include selective utilisation, expulsion and absorption of ejaculates (Villavaso 1975a; Boggs and Gilbert 1979; Gwynne 1984; Birkhead and Møller 1993).

The double mating experimental paradigm employed in studies of sperm competition typically allows the second male to copulate with a female directly after the first (Parker 1970b; Smith 1984), and effectively prevents females from influencing the storage and use of the first ejaculate before the second male can also influence its fate (Ward 1993). It is therefore not surprising that the literature contains many documented examples of flushing or direct removal of sperm by males (e.g. Smith 1984; Birkhead and Hunter 1990) but relatively few examples of female control of

paternity and the likely mechanisms involved (Knowlton and Greenwell 1984; but see Walker 1980; Ward 1993; Eberhard 1996).

Unlike those of *P. diplodontus*, the gonopods of *A. uncinatus* reveal no obvious morphological structures that could function in ejaculate removal (see Figs 2.5 and 2.19). The spines on the distal ends of the coxite do not enter the vulval sacs and the distal ends of the telopodite arms are smooth and tapering, unlike the serrated plates of *P. diplodontus*. However, the bursae are situated deeply within the vulval sacs, suggesting that the telopodites may play a role in ejaculate translocation.

In this study, radioactive labelling techniques are used to examine patterns of ejaculate transfer in *A. uncinatus* and to test whether sperm storage is affected by a temporal delay between successive matings. Observed patterns are discussed in the context of gonopod functional morphology and female mediation of competition. A simple genital manipulation is performed to elucidate the role of the gonopod telopodite.

6.2 Methods

Animals were collected, housed and labelled with tritiated thymidine ([methyl-³H]thymidine; 85 Ci/mmol, Amersham, UK) as in Chapter 4. It was assumed that ejaculates were evenly distributed with the label.

6.2.1 Mating sequences

The mating sequences performed by *A. uncinatus* are summarised in Table 6.1.

a. Basic mating sequences

As in *P. diplodontus*, initially two series of basic experiments were conducted, each comprising two mating sequences. In the first sequence, females were mated first with a labelled male and then with an unlabelled male (L-UL), and the results were used to quantify the ejaculate contribution of the first male. In the second sequence, the order was reversed, and females were mated first with an unlabelled and then with a labelled male (UL-L), thus quantifying the contribution of the second male. Two experimental series were conducted in order to establish a starting point for examining temporal

TABLE 6.1 *A. uncinatus* mating sequences. L = labelled male; UL = unlabelled male. (M) following either of these indicates that the male's gonopods were manipulated.

<i>mating sequence</i>	<i>purpose</i>
UL only	control
L only (0 & 24 hour)	ejaculate contribution of male 1 - single mating
L - UL (0 & 24 hour)	ejaculate contribution of male 1 - double mating
UL - L (0 & 24 hour)	ejaculate contribution of male 2 - double mating
L - UL (M)	role of telopodite in displacement
L (M)	role of telopodite in placement or self sperm displacement

changes in sperm precedence between matings. In the first series, matings followed each other immediately and in the second, the delay between matings was 24 hours. Females were also mated once with labelled males (L) to quantify ejaculate transfer in single matings.

b. Manipulation sequences

An additional series of experiments was conducted to elucidate gonopod function. Two mating sequences involving a gonopod manipulation were performed. Gonopods of males to be manipulated were everted with forceps and the left telopodite was severed close to its point of emergence from the coxite (Fig. 6.1). During copulation with manipulated males, the right vulva of females was penetrated by the gonopod with the missing telopodite.

In the first mating sequence, females were mated with manipulated labelled males only (L(M)) to elucidate the function of the telopodite in self-sperm placement and displacement. In the second, they were mated first with normal labelled and then with unlabelled manipulated males (L-UL(M), 0 hour delay) to elucidate the role of the telopodite in rival sperm manipulation.

c. Controls

As mentioned in Chapter 4, vulvae from females that were mated with males injected with 50 μ l sterile distilled water were used as controls. The range of dpm values obtained was consistent with values for background counts (dpm with background

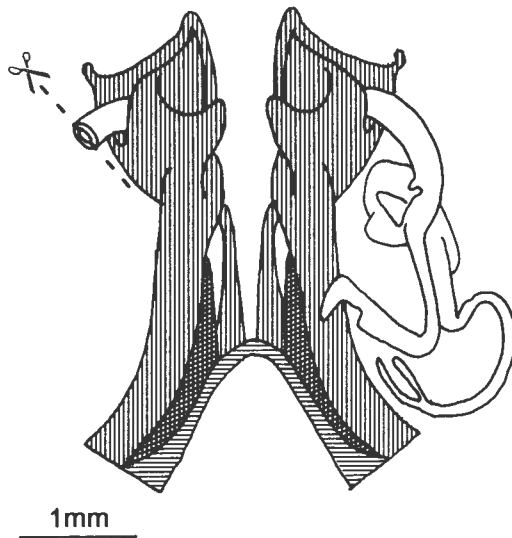


FIGURE 6.1 *A. uncinatus* gonopods illustrating the point at which the left telopodite was severed.

||| = coxite; □ = telopodite; ▨ = sternite.

subtracted: 1.9 - 9.5 dpm, $n = 4$).

Following mating, females were sacrificed in ethyl acetate killing jars and their vulvae were processed for scintillation counting (as in Chapter 4).

6.3 Results

6.3.1 Basic mating sequences

When the second mating immediately followed the first (Experiment 1), there was no significant difference in dpm between the first and second mating sequence (L-UL vs UL-L: Two-tailed Mann-Whitney U-test, $U = 64$, $P = 0.71$, $df = 14$, and see Table 6.2), and also no difference between either of these and single matings (L only vs L-UL: $U = 107$, $df = 18$, $P = 0.153$, L only vs UL-L: $U = 88$, $df = 18$, $P = 0.07$). These results indicate that a second mating does not affect the ejaculate of the first male, and that the presence of the first ejaculate does not affect storage of the second ($V_2 = 0.5$).

Preliminary investigations into vulval capacity are inconclusive (Fig 6.2) and therefore the effect of the presence of more than two ejaculates on subsequent storage is not clear.

TABLE 6.2 Mean (\pm 1 s.d.) disintegrations per minute and the proportion of ejaculates placed at the bottom of the vulvae for the two series of basic double mating experiments. (L = labelled male; UL = unlabelled male). A comparison between dpm values of all mating sequences yielded significant differences (Kruskal Wallis $H = 18.18$, $df = 5$, $P = 0.003$). However, when the data from the L-UL (24) sequence were excluded, dpm from the remaining mating sequences were not significantly different (Kruskal-Wallis L, L-UL (0), UL-L (0) and UL-L (24): $H = 3.7$, $df = 3$, $P = 0.296$).

hours between matings	mating sequence	mean dpm (\pm s.d.)	<i>n</i>
-	L	839.1 (\pm 312.6)	11
0	L-UL	1131.9 (\pm 494.1)	8
0	UL-L	1317.6 (\pm 660.8)	8
24	L-UL	467.7 (\pm 342.3)	6
24	UL-L	1012.4 (\pm 547.8)	7

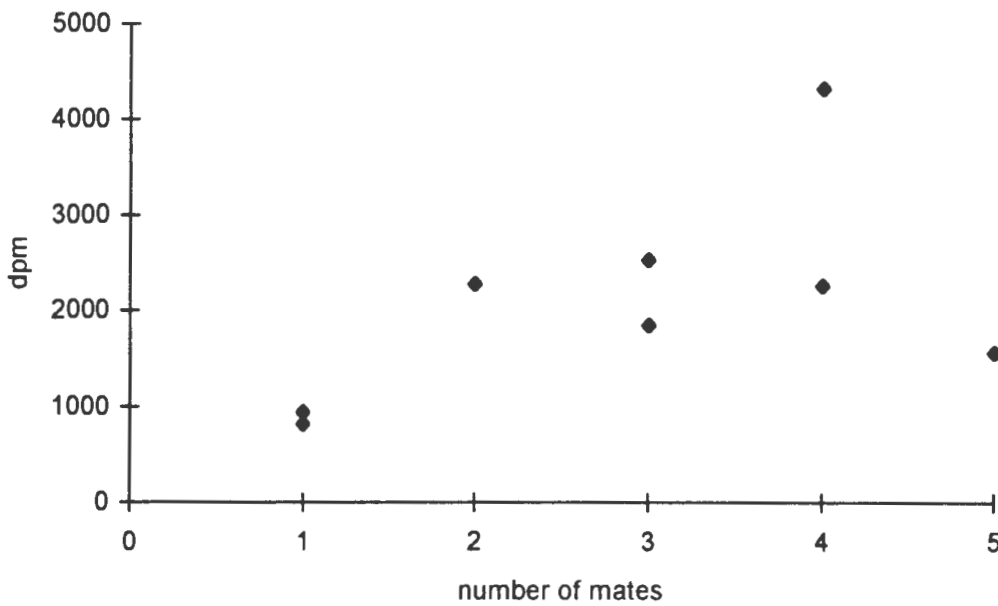


FIGURE 6.2 Preliminary investigation into *A. uncinatus* vulval capacity. Dpm (disintegrations per minute) are plotted vs number of successive matings with labelled males ($r = 0.53$, $P = 0.17$, $n = 8$).

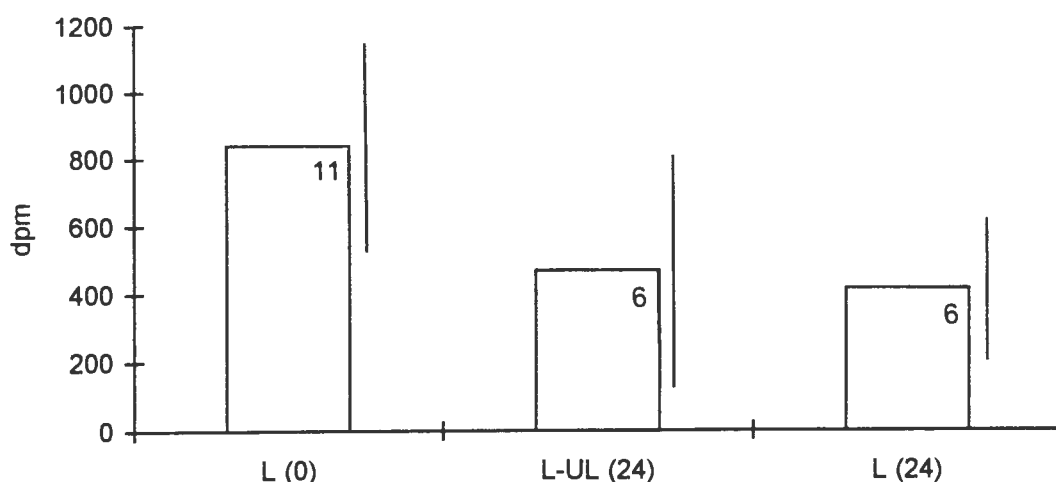


FIGURE 6.3 Mean dpm values for L(0), L-UL(24) and L(24) mating sequences. Vertical lines indicate standard deviations. Numbers on bars indicate sample sizes.

A delay of 24 hours between the first and second mating (Experiment 2) yielded a significantly lower dpm for the first mating sequence (L-UL (24) vs UL-L (24), $U = 26$, $P = 0.03$, $df = 11$, and see Table 6.2). These results suggest that when there is no delay between matings, females store an equivalent volume of both males' ejaculates, but when a 24 hour delay is effected, there is a significant reduction in the contribution of the first male ($V_2 = 0.71$). The reduction could either be a consequence of displacement by the second male to mate, or a consequence of absorption or expulsion of part of the first ejaculate by the female over the 24 hour delay.

To distinguish between these alternative explanations, additional matings were conducted in which females ($n = 6$) were mated once with labelled males, and only dissected 24 hours following this copulation. If the reduction in dpm following the 24 hour delay was a consequence of a female process, then dpm from these single matings would be equivalent to that of dpm from the L-UL mating sequence of Experiment 2. If dpm reductions were a consequence of a male process, then counts would be equivalent to the L(0) mating sequence of this experiment. These results support the female process hypothesis (L-UL (24) vs L (24), $U = 38$, $P = 0.93$, $df = 10$; Fig. 6.3).

Anecdotal evidence suggests that the reduction in dpm was an effect of a reduction in ejaculate volume and not simply a reduction in isotope.

TABLE 6.3 Proportion dpm placed in bottom vulval component.

<i>delay between matings</i>	<i>mating sequence</i>	<i>n</i>	<i>% dpm at bottom (±s.d.)</i>
0	L - UL	8	55.4% (± 4.6%)
0	UL - L	8	54.7% (± 3.8%)
24	L - UL	6	58.1% (± 4.0%)
24	UL - L	5	59.3% (± 8.0%)

1. If the reduction in dpm was the result of isotope loss, similar reduction patterns would be expected across all species due to similar vulval morphologies at the histological level. These were not observed in *P. diplodontus*, see Chapter 5.

2. Ejected sperm secretions have previously been found at the spermathecal openings of a female with no recent mating history (Blower 1985; Tadler 1996). This has been suggested to constitute evidence for sperm ejection (Tadler 1996).

3. The vulvae of females sacrificed immediately (n=5) and 24 hours following copulation (n=4) were processed for histology and sectioned (see Chapter 3 for histological processing methodology). Free sperm occurred within the vulval sacs of *A. uncinatus* immediately following copulation but, within 24 hours, most sperm were found within the spermathecae. The reduction in free sperm in the vulvae 24 hours following copulation substantiates a reduction in ejaculate volume. While it is possible that free sperm were incorporated into the spermatheca, this interpretation is not consistent with the reduction in dpm.

6.3.2 Ejaculate distribution

Irrespective of the time delay between matings, the proportion of dpm at the bottom of the vulvae was the same for males in both mating sequences (Table 6.3), suggesting that ejaculates are not stratified between top and bottom vulval components (on a macro level). Most (54.27 - 59.33%) of the ejaculates of both males were placed in the vulval sac component containing the bursa (the bottom).

The implications of this distribution for sperm precedence are unclear: although there is no stratification on a macro level between top and bottom components, this methodology cannot detect microlayering, and therefore cannot exclude potential

fertilisation benefits accrued from microstratification of ejaculates either within the vulvae or the spermathecal ampullae. Because there is typically a long delay between insemination and oviposition, mixing of ejaculates within the vulvae/spermathecae is equally possible. As in *P. diplodontus*, selective ejaculate storage by females cannot be excluded.

6.3.3 Manipulation sequences

In *A. uncinatus*, the telopodite is implicated in the translocation of ejaculates to the vulvae. In the L(M) sequence, dpm values for the right vulvae (that interacted with the manipulated gonopod) were significantly lower than those of the left (Wilcoxon test left vs right: $T = 21$, $n = 6$, $P < 0.05$). Interestingly, there were no differences in the proportion of total dpm placed at the bottom of the vulvae, indicating that although the telopodite does affect ejaculate transfer, it does not play a role in ejaculate movement to the bottom of the vulvae ($T = 4$, $n = 6$, $P = 0.855$; Table 6.4).

Rival sperm was unaffected by telopodite function: dpm values of left and right vulvae from the L-UL(M) mating sequence were the same (Wilcoxon test: $T = 14$, $n = 7$, $P = 1$) (Table 6.4).

6.3.4 Copulation duration

The duration of copulation did not differ significantly between first and second matings (Mean copulation duration first mating = 100.6 ± 36.8 mins, $n = 28$; mean copulation duration second mating = 96.7 mins ± 44.4 , $n = 28$; Wilcoxon test: $P = 0.316$, $n = 28$, $T = 215.5$), and was not affected by the interval between matings (no significant differences between relative copulation duration of first and second matings, 0 hr vs 24 hr delays: Mann-Whitney: $U = 240.5$, $P = 0.14$, $df = 25$).

Interestingly, copulation duration was affected by the telopodite manipulation, with males having manipulated telopodites copulating for shorter periods than those with intact gonopods (Mann-Whitney: $U = 2443$, $P = 0.022$, $df = 71$). Copulation duration of males with intact gonopod telopodites = 217.5 ± 36.8 , $n = 62$, copulation duration of males with manipulated gonopod telopodites = 157.9 mins ± 59.1 , $n = 11$. The same was true of *P. diplodontus* (see previous chapter). (Note that for *A. uncinatus*, the

TABLE 6.4 Proportion dpm placed in left and right vulvae, manipulation sequences

<i>mating sequence</i>	<i>dpm</i>	<i>mean</i>	<i>s.d.</i>	<i>n</i>
L(M)	proportion left	0.69	0.07	4
	proportion right*	0.31	0.07	4
	proportion left bottom	0.61	0.17	4
	proportion right bottom*	0.58	0.08	4
L- UL(M)	proportion left	0.51	0.11	7
	proportion right*	0.49	0.11	7

* vulval sac penetrated by the gonopod with telopodite removed.

mean copulation duration of males with unmanipulated gonopod telopodites differs from the mean obtained in the basic mating sequences. The telopodite manipulation experiments were discrete from the series of basic mating experiments, and were conducted on a different population of *A. uncinatus* millipedes).

6.3.5 Covariation in dpm

As in *P. diplodontus*, the absence of a relationship between male body mass and dpm (disintegrations per minute) indicated no relationship between male body mass and ejaculate volume and also no difference in the intensity of radioisotope labelling of ejaculates of males of different sizes. (*A. uncinatus*: Pearson's correlation coefficient: $r = 0.18$, $P = 0.53$, $n = 14$, two-tailed test). Predictably there was no relationship between dpm and copulation duration ($r = 0.14$, $P = 0.63$, $n = 14$, ejaculates are transferred at the beginning of copulation, see Chapter 4), and female body mass and dpm did not covary ($r = 0.49$, $P = 0.07$, $n = 14$).

Interpretation of the data

Female mating history prior to the experiments was not known and the possible presence of sperm from previous matings is not accounted for. Therefore, as in *P. diplodontus*, sperm volumes give a measure of $V_{(x)}$ and $V_{(x+1)}$, hereafter referred to as V_1 and V_2 respectively.

6.4 Discussion

Most previous studies on arthropods that directly addressed the effect of temporal separation between successive matings on P_2 have shown a *decrease* in P_2 over time (e.g. Radwan 1991; Ward 1993). The volume of sperm displaced by the second male to mate has also been shown to decrease with a temporal delay between successive matings in the beetle, *Tenebrio molitor* (Gage 1992). These results were all attributed to the migration of sperm of the first male to regions of the female storage organs from where they could not easily be displaced. P_2 has also been shown to decrease as a result of a change in ejaculate distribution patterns - an initial benefit accrued by the last male due to ejaculate stratification may be lost when ejaculates mix (Zeh and Zeh 1994).

In *A. uncinatus*, V_2 increased with the delay between matings. Neither male had a competitive advantage when matings followed each other immediately (Experiment 1) and last male superiority (volumetrically) occurred when a 24 hour delay was imposed (Experiment 2). If ejaculates are homogeneously distributed with sperm and used for fertilisation in proportion to their volumes, this result suggests a temporal *increase* in P_2 similar to that of *D. melanogaster* (Boorman and Parker 1976; Gromko *et al.* 1984) in which P_2 increased with a delay between matings and was related to a reduction in the first male's sperm (Gromko *et al.* 1984). In the melon fly *Dacus cucurbitae*, an increase in P_2 was attributed to sperm mortality (Tsubaki and Yamagishi 1991; Yamagishi *et al.* 1992). This is an unlikely explanation for *A. uncinatus* as mortality would not have affected dpm.

Reduction in sperm numbers has been associated with direct removal by males (e.g. Waage 1979a, see Chapter 1) and the stimulation of females to discard previous ejaculates (Villavaso 1975a; Etman and Hooper 1979; Davies 1983; 1985; Miller 1987b; Eberhard 1996). Because the reduction in dpm in *A. uncinatus* occurred without a second mating, and therefore in the absence of direct male mediation, neither of these hypotheses are supported.

Previous studies have shown females to manipulate ejaculates through selective utilisation (Birkhead and Møller 1993), expulsion (Villavaso 1975a; Gromko *et al.* 1984; Otronen 1990; Birkhead and Hunter 1990) and absorption (Gwynne 1984; Radwan and Witalinski 1991). No oviposition occurred during the experimental period and therefore loss of ejaculates through utilisation for fertilisation can be excluded. It

is therefore concluded that the reduction in ejaculate volume was a consequence of either ejaculate absorption or expulsion. The latter explanation is supported by genital grooming by females following copulation: in addition to cleaning, grooming has been associated with feeding on vulval contents (Mukhopadhyaya and Bhakat 1983; Tadler pers. com.) and excess sperm may thus constitute a nuptial gift (Thornhill 1976; Gwynne 1984).

Consistent with the finding that the reduction in dpm is a female-mediated process, is the lack of a role of the telopodite in displacement (no differences between left and right vulvae in L-UL(M) mating sequence). However, and unlike *P. diplodontus*, the telopodites are implicated in ejaculate placement. Consequently telopodite morphology may reflect the evolution of devices to effectively place sperm (see Chapter 2). Interestingly, in spite of the location of *A. uncinatus*' bursae in deep vulval sacs, the telopodites are not implicated in ejaculate translocation to the bottom of the vulvae.

Stimulation of females to accept ejaculates was previously cited as a possible reason for prolonged genital contact in *A. uncinatus* (see Chapter 4). The expulsion of ejaculates by females over time indicates an additional explanation: *A. uncinatus* males may prolong copulation to prevent females from discarding/consuming their ejaculates post insemination (Alcock 1994). This explanation is consistent with an increase in copulation duration according to the mate guarding hypothesis: the benefit of ejaculate storage assurance may outweigh the cost of not securing additional matings when females are scarce.

Insemination occurs early during copulation (Barnett and Telford 1994, Chapter 4) and ejaculates are not stratified (on a macro level) within the vulvae. Under these conditions, selection for sperm removal would not be favoured because males would risk partial self-sperm removal (Parker G.A. in Birkhead and Møller 1993; Chapter 4). Flushing may also not be mechanically possible due to the distance of the stores from the gonopore. Consequently, in *A. uncinatus*, males may compete through ejaculate dilution (Parker 1970a; Walker 1980; Birkhead and Møller 1992; Eady 1995). Although not an optimal means of ensuring paternity, the resultant dilution of the ejaculate of the first male to mate is effective in that it reduces the predicted fertilisation success of the first male by at least 50%.

A superficial analysis of the mating system of *A. uncinatus* suggests it to be male dominated - males control the duration of copulation (Telford and Dangerfield 1993b; 1994), copulation has been interpreted as a form of mate guarding (Telford and Dangerfield 1990a) and the formation of triplets (in which a single male associates himself with a copula pair and remains attached until the pair separates and he can copulate with the female; Telford and Dangerfield 1990a, b; pers. obs.) imply little mate choice for females. The use of the non-gametic component of ejaculates as mating plugs has also been suggested (see Chapter 3). However, where females have little choice between mates or copulations are forced, some physiological or anatomical control of paternity may still be feasible (Birkhead and Møller 1993). These results show that females are responsible for ejaculate depletion during the temporal delay between matings, and this manipulation of ejaculates post copulation may afford females the ultimate control of offspring paternity. Where sperm storage is regulated, as has been a suggested function of the bursal muscles (Chapter 3), expulsion and partial expulsion of ejaculates could be a mechanism by which females exclude undesirable sperm while collecting a diversity of sperm from "acceptable" males.

CHAPTER SEVEN

THE MECHANISMS OF COMPETITION IN THE SPIROSTREPTIDA

7.1 Introduction

By combining studies of genital morphology and function with direct ejaculate labelling, processes of ejaculate competition and cryptic female choice were elucidated in the Spirostreptida. The key results of this work are summarised in Table 7.1. Odontopygidae data are preliminary and trends are not clear (see Appendix D). Consequently, the discussion that follows focuses primarily on the Harpagophoridae and the Spirostreptidae.

7.2 Processes of sperm transfer

Cognisance of the mechanisms of competition depends on an understanding of the processes of sperm transfer and subsequent storage.

Spirostreptida males transfer sperm from the penes to the vulvae via complex, species-specific gonopods that comprise three components, the coxite, telopodite and sternite. Sperm are transferred via the coxites of the gonopods. In the Spirostreptidae, the telopodites are also partly implicated in ejaculate transfer. This function may be associated with the complex telopodite retraction-release cycles: in the Harpagophoridae and the Odontopygidae, telopodites are only partially retracted and their distal ends do not make contact with the coxites. In the Spirostreptidae, full retraction brings the distal telopodite in contact with the coxal spines, and this contact may facilitate the transfer of sperm from the coxite to the telopodite. In this context, the evolution of coxal spines in the Spirostreptida is explicable in terms of temporary ejaculate suspension.

TABLE 7.1: Summary of results.

	Harpagophoridae	Spirostreptidae	Odontopygidae	Chapter
Gonopod morphology	Elaborate			2
Gonopod functional morphology	Complex retraction-release cycles performed by the telopodite			2
Do telopodites touch the coxites when retracted?	No - femoral spine restricts movement	Yes	No - telopodite spins about own axis	2
Sperm morphology	non motile			2
Bursal morphology	Sperm stored in spermathecal ampullae that are associated with glands and muscles (selective storage?). Ova emerge from under an operculum at the anterior end of the spermatheca.			3
No. stores	Few	Many	Many	3
Depth of vulval sac from gonopore	Deep	Deep	Deep	3
Proximity of stores to gonopore	Close	Far	Far	3
Female mechanism of syn- and/or postcopulatory ejaculate discrimination ?	Yes	Yes	Yes	3
Vulval capacity	Reached with single ejaculates	Not reached with single ejaculates		5, 6
Do distal telopodites enter the spermathecal ampullae?	No	No	No	2, 3
Do distal telopodites have access to the spermathecal furrow?	Yes	Probably	Unknown	2, 3
Timing of insemination	Early. Ejaculates move away from gonopore and are partially lost shortly after transfer		Early in <i>C. digitatus</i>	4, D
Do telopodites function in ejaculate transfer?	No	Yes	No	5, 6, D
Are prior ejaculates displaced?	Yes, 64.52% are removed	Yes, displacement by female with temporal delays	No displacement from vulvae	5, 6, D
Do telopodites function in rival ejaculate displacement?	Yes, partly. Only 26.46% by telopodite	No	No	5, 6, D
V_2	0.62	0.5 with no delay, 0.7 with delay	<i>C. digitatus</i> = 0.5 <i>Spinotarsus</i> sp. 1 <0.5?	5, 6, D
Effect of temporal delays between matings	No effect	Reduction in prior sperm	No effect	5, 6, D
Does the telopodite manipulation affect copulation duration?	Yes	Yes	No (but n = 4)	5, 6, D

The minimal role that the telopodites play in sperm transfer has interesting implications for the role of the telopodite canal because it demonstrates that the canal does not transport ejaculates from the base of the coxae, or ejaculate components from the coxal gland to the vulvae, as previously speculated (Attems 1928; Demange 1959).

Females store sperm within bursae in their vulval sacs, in blind-ending spermathecal ampullae from which muscle bands fan out to the bursal walls. Ova emerge from the oviduct from under a bursal operculum and are fertilised as they pass through the bursal furrow that gives rise to the apodemic tube and the spermathecal ampullae. It is not clear whether ejaculates are stratified or mixed within the spermathecal ampullae but, owing to the delay between insemination and oviposition, mixing is probable. Ejaculate stratification would confer an advantage to the last ejaculate entering the stores. With mixing, paternity assurance would be proportional to ejaculate representation.

Distal telopodites were broader than the apodemic tube in all species examined, indicating that they do not have direct access to stored sperm. Because the telopodites do not enter the stores directly, and sperm are non-motile, the process by which the sperm enter the stores is probably female-mediated. Two possible mechanisms, bursal muscle pumping and inward flow systems, have been suggested (Seifert 1932; Tadler 1993).

Because it affects processes of sperm transfer, studies of the mechanisms of competition should include a measure of the timing of insemination. In the Spirostreptida, insemination occurs almost instantaneously, shortly after the onset of copulation. This places obvious constraints on subsequent processes of ejaculate manipulation: males cannot manipulate rival ejaculates without also affecting their own.

7.3 Mechanisms of competition

Separating the effects of cryptic female choice from male-mediated sperm competition is critical when measuring the impact of these processes on patterns of sperm precedence. This study attempted to identify these processes of competition, and quantify their influence on processes of ejaculate transfer and subsequent storage. In all three families of Spirostreptida millipedes, several male- and female-mediated mechanisms affect V_2 (see section 5.3).

7.3.1 Sperm competition

As mentioned in Chapter 1, Parker (1970a) identified two sets of opposing adaptations that evolve to increase ejaculate paternity assurance - those that pre-empt previous ejaculates and those that prevent their pre-emption. These include indirect "strategies" to induce females to accept, store and utilise their sperm, or discard those of previous males.

a. Pre-emption of previous ejaculates

Where insemination is early, males cannot manipulate rival ejaculates without also affecting their own. This, combined with the inaccessibility of the spermathecal ampullae, implies that absolute removal of rival ejaculates is not possible in the Spirostreptida. It is suggested that two different pre-emption mechanisms combine to produce the observed patterns of V_2 ; (a) competition through dilution within the spermathecal ampullae and (b) competition through ejaculate flushing in the rest of the vulvae, particularly the bursal furrow.

The most simple type of sperm competition, dilution, occurs when ejaculates are simply added to those already within the stores. Because Spirostreptida males do not have direct access to sperm stored within the ampullae, this is the only mechanism by which they can compete with stored sperm. With ejaculate mixing, paternity assurance is proportional to ejaculate representation. The relationship between ejaculate volume transferred and stored is not clear but if these are proportional, selection would favour the evolution of larger ejaculates. The seemingly oversized genitalia of *Spinotarsus* sp. 1 (see Appendix D) were interpreted in this context.

The stimulation of females to store ejaculates may facilitate the uptake of ejaculates in close proximity to the stores. Consequently, males would be selected to remove unstored rival ejaculates from the vicinity of the spermathecae. This may be achieved through ejaculate flushing (see section 4.4). This hypothesis is supported by the transfer of larger ejaculates than remain within the vulvae following copulation. The effect of bursal muscle contraction on processes of ejaculate flushing is not clear, but contraction may allow later males to flush rival sperm from the openings of the

spermathecal ampullae as well. In this context, females may mediate displacement from otherwise inaccessible regions.

The distal telopodites reach the bursal furrows in *A. uncinatus* and *P. diplodontus*, and may play a role in displacing stored sperm or mating plugs (see section 3.4). Where stores are in close proximity to the gonopore, displaced sperm may be flushed from the vulvae completely (*P. diplodontus*). The telopodite plays a role in this process of ejaculate removal and it may function to dislodge ejaculates from the furrow. Where stores are located deeply within the vulvae (*A. uncinatus*, and the Odontopygidae), although displacement of sperm from the furrows may be effected, removal from the vulval sacs is not possible.

b. Anti-preemption

There is apparently no refractory period between Spirostreptida matings, and males would be selected to protect their gametic investment in females, especially in populations with male-biased OSR's.

In all of the Spirostreptida species examined in this study, the duration of copulation far exceeds the time required for sperm transfer. The functional significance of this may be found in mate guarding, as suggested by Telford and Dangerfield (1991a) and supported by an increase in the duration of copulation with increased male-bias in the OSR (*A. uncinatus*, Telford and Dangerfield 1991a). Prolonging the duration of copulation may prevent later males from flushing ejaculates from the bursal furrow (*A. uncinatus* and *P. diplodontus*) and prevent females from discarding ejaculates (*A. uncinatus*). A third function related to female stimulation is also suggested (see c below).

The non-gametic component of Spirostreptidae ejaculates may function to prevent later ejaculates from entering the spermathecae. In several Spirostreptidae, the granules that are transferred with ejaculates accumulated in the bursal furrow, and apparently block access to the spermatheca. This may reduce the impact of ejaculate flushing by subsequent males, or prevent their ejaculates from being taken into the stores.

7.3.2 Cryptic female choice

a. Control over ejaculate storage and displacement

Irrespective of the exact process, female mediation over processes of sperm translocation into the spermathecal ampullae (see above) provides a mechanism of syn- and postcopulatory discrimination between ejaculates. Females may selectively store ejaculates of preferred males by regulating sperm transport into the spermathecal ampullae. Similarly, they may control the displacement of stored ejaculates: males do not have direct access to the spermathecal ampullae and, as mentioned above, displacement may be contingent upon female co-operation.

Unstored ejaculates are displaced from the vulvae in both *A. uncinatus* and *P. diploodontus*. The mechanisms of displacement differ; it is female-mediated in *A. uncinatus* in which ejaculates are displaced in the interval between matings. Displacement is male-mediated in *P. diploodontus* but the proximity of the stores to the gonopore in this species, and the inability of males to enter the spermathecal ampullae directly, suggests female incitement of direct male competition (see later).

b. Internal courtship

In the Spirostreptida, the telopodites are the largest and most complex gonopod components. Although in some species they are implicated in processes of ejaculate transfer (*A. uncinatus*) and removal (*P. diploodontus*), general gonopod complexity is not accounted for by selection for efficient transfer or displacement devices. Pleiotropy does not account for gonopod evolution either, because the association of severed telopodites with reduced copulation durations (*A. uncinatus* and *P. diploodontus*) suggests some functional significance to telopodite morphology. An alternative explanation may be found in cryptic female choice: gonopods may have evolved to indirectly improve ejaculate transfer efficiency by functioning to stimulate females to accept ejaculates. In this context both the elaborate telopodite retraction-release cycles and prolonged copulations are explicable. The cues on which storage decisions are based are unclear. There is no indication that preferences are associated with male body size, but because ejaculate micro-distribution patterns within the spermathecae are not known, these results remain inconclusive.

7.4 Evolution of the Genitalia

7.4.1 Vulvae

In the Glomerida, Polyzoniida and Chordeumatida, vulvae are located outside the body of the female, whereas at resting position in the Polydesmida, Spirobolida, Julida and Spirostreptida, they are retracted into the body. These differences have been related to burrowing activity: in millipedes that burrow, sternites and pleurotergites are fused and vulval sacs are held within the body, apparently to protect them (Tadler pers comm.). Differences in vulval morphology may thus have initially evolved under natural selection. Because they have implications for the mechanisms of sperm competition, subsequent adaptations may have evolved in this context.

Basic bursal morphology is conserved within the Spirostreptida and apparently within the Eugnatha (Chordeumatida, Tadler 1993; Polydesmida, Brölemann and Lichtenstein 1919; Spirobolida, pers. obs.; Julida, Tadler 1996). However, bursae differ in their proximity to the gonopore and in spermathecal capacity.

In the Spirostreptida, bursae are located in deeply invaginated vulval sacs. In most cases, these restrict male access to the sperm stores and may be a consequence of female adaptations for control over processes of ejaculate storage. The evolution of deep vulval sacs concurs with the evolution of the larger, more mobile telopodites that characterise the Spirostreptida. If vulval sac deepening preceded gonopod lengthening, the lack of bursal hairs in the Spirostreptida is also explicable. Hairs occur on the bursae of the Chordeumatida, Julida and Spirobolida (e.g. Kurnik and Thaler 1985; Tadler 1993; pers. obs.) and have previously been ascribed a sensory function (Tadler 1993). Sensory hairs would have no functional significance where bursae are located deeply within the vulval sacs such that the gonopods could not reach them, and consequently may have been lost in the Spirostreptidae.

Although Harpagophoridae bursae are located in deep vulval sacs, due to the evolution of bursal plates they almost protrude from the gonopores. The shallow position of the Harpagophoridae bursa may represent the secondary evolution of accessible stores by females, possibly to promote direct competition between competing males. Concurring with this close proximity to the gonopore is a reduction in spermathecal capacity. In *P. diploidontus*, only five spermathecal chambers occur and vulval capacity is limited to

single ejaculates. In contrast, in both *A. uncinatus* and *S. fiedleri complicans* in excess of thirty chambers occur. In spite of females ejecting sperm, in *A. uncinatus* at least two and possibly four ejaculates can be accommodated. If spermathecal capacity is related to the relative importance of storing sperm, then this indicates that in the Harpagophoridae, storage may be less significant.

7.4.2 Gonopods

Spirostreptida coxites are species-specific and although they function in processes of ejaculate transfer, the functional significance of interspecific morphological differences is not clear. Coxites do not move during copulation and a close association between them and the vulvae, similar to that found in the Chordeumatida (Tadler 1993), cannot be excluded. A stimulatory role in this context may account for their complexity.

Spirostreptida telopodites are generally not implicated in processes of ejaculate translocation. However, their potential role in internal courtship (which is related to bursal morphology and the ability of females to effect syn/postcopulatory discrimination) is common to all Spirostreptida families. It is therefore suggested that telopodites evolved initially in the context of internal courtship and were subsequently modified in some cases (e.g. *Alloporus* spp, Spirostreptidae) via selection for efficient ejaculate translocation. This finding concurs with Tadler (1996) who suggested that gonopod function evolved several times in the Diplopoda.

7.5 Predicting the mechanisms of competition from genital morphology

Slight inter-generic differences between gonopod telopodites may impact on their functional morphology (e.g. telopodite mobility is less restricted in *Harpagophora* spp than *Poratophilus* spp) and affect their interaction with the vulvae (e.g. scoops on the distal telopodites of *Orthoporoides* spp vs tapering flagellae in *Alloporus* spp). However, due to the minimal impact that telopodites have on processes of ejaculate transfer and displacement, these differences may be inconsequential to the mechanisms of competition. Processes of displacement identified in this study were, however, contingent upon vulval morphology. It is therefore suggested that, in the Spirostreptida, vulvae may be better predictors of the mechanisms of competition than gonopods.

7.6 Conclusions

Disentangling male and female sexually selected processes remains a major challenge in evolutionary biology. By combining cognition of genital morphology with direct ejaculate labelling, mechanisms that potentially dictate processes of sperm transfer and subsequent storage were elucidated in the Spirostreptida. Although many of these require clarification, this study illustrates both the intensity and complexity of selection at the gametic level, and hence the usefulness of studying the mechanisms of sexual competition. The application of this approach to other polygynandrous taxa could facilitate a better understanding of the significance of syn- and postcopulatory intrasexual competition and female choice.

APPENDIX A

CLADISTIC CLASSIFICATION OF THE DIPLOPODA (AFTER ENGHOFF 1984)

Class Diplopoda

Subclass Penicillata

Order Polyxenida

Subclass Chilognatha

Infraclass Pentazonia

Order Glomeridesmida

Order Sphaerotheriida

Order Glomerida

Infraclass Helminthomorpha

Subterclass Colobognatha

Order Platydesmida

Order Siphonophorida

Order Polyzoniida

Subterclass Eugnatha

Superorder Nematophora

Order Stemmiulida

Order Callipodida

Order Chordeumatida

Superorder Merocheta

Order Polydesmida

Superorder Juliformia

Order Spirobolida

Order Julida

Order Spirostreptida

Family Spirostreptidae

Family Harpagophoridae

Family Odontopygidae

APPENDIX B

ANIMAL COLLECTION SITES

species	collection site	co-ordinates	date
FAMILY HARPAGOPHORIDAE			
<i>Harpagophora levis</i>	Ngutu, KwaZulu	28°10'S, 30°50'E	October - November 1992
<i>Harpagophora polyodus</i>	Clanwilliam, Western Cape	31°50'S, 18°50'E	September 1996
<i>Poratophilus dipodontus</i>	Moreletta, Pretoria Nelspruit, Mpumalanga	25°40'S, 28°12'E 25°20'S, 31°05'E	October - February 1993-4
<i>Poratophilus punctuatus</i>	Port Elizabeth , Eastern Cape	34°10'S, 26°20'E	March 1994
<i>Poratophilus similus</i>	White River, Mpumalanga	25°50'S, 30°40'E	October - November 1992
<i>Poratophilus</i> sp.1	Kruger National Park	25°05'S, 31°42'E	January 1994
<i>Poratophilus</i> sp.2	Kruger National Park	25°05'S, 31°42'E	January 1994
<i>Poratophilus</i> sp.3	Hwange, Zimbabwe	18°35'S, 26°20'E	January - February 1993
<i>Poratophilus</i> sp.4	Kruger National Park	25°05'S, 31°42'E	October - November 1992
<i>Poratophilus</i> sp.5	Kruger National Park	25°05'S, 31°42'E	October - November 1992
<i>Poratophilus</i> sp.6	Kruger National Park	25°05'S, 31°42'E	October - November 1992
<i>Poratophilus</i> sp.7	Kruger National Park	25°05'S, 31°42'E	October - November 1992

species	collection site	co-ordinates	date
FAMILY SPIROSTREPTIDAE			
<i>Alloporus bilobatus</i>	Kruger National Park	25°05'S, 31°42'E	October - February 1993-4
<i>Alloporus castanaes</i>	Piggs Peak, Swaziland	25°50'S, 31°12'E	October - November 1992
<i>Alloporus falcatus</i>	Big Bend, Swaziland	27°10'S, 31°40'E	October - February 1993-4
<i>Alloporus flavifilis</i>	Kruger National Park	25°05'S, 31°42'E	January 1994
<i>Alloporus flavifilis</i> sub sp.	Kruger National Park	25°05'S, 31°42'E	January 1993-4
<i>Alloporus levigatus</i>	Moreletta, Pretoria	25°40'S, 28°12'E	October - February 1993-4
<i>Alloporus rugifrons</i> sub sp.	Kruger National Park	25°05'S, 31°42'E	April 1993
<i>Alloporus transvaalensis</i>	Kruger National Park	25°05'S, 31°42'E	October - February 1993-4
<i>Alloporus uncinatus</i>	Mazowe, Zimbabwe	18°22'S, 28°17'E	January - February 1993
<i>Alloporus</i> sp.1	Kruger National Park	25°05'S, 31°42'E	October 1992, January 1994
<i>Alloporus</i> sp.2	Kruger National Park	25°05'S, 31°42'E	October - November 1992
<i>Bicoxidens matabele</i>	Chimanimani, Zimbabwe	18°30'S, 32°42'E	January - February 1993
<i>Orthoporoides pyrhocephalus</i>	Grahamstown, Eastern Cape	33°15'S, 26°20'E	March 1991
<i>Orthoporoides tabulinus</i>	Kruger National Park	25°05'S, 31°42'E	October - February 1993-4
<i>Trienostreptus unciger</i>	Gaborone, Botswana	24°50'S, 25°55'E	April 1993

species	collection site	co-ordinates	date
FAMILY ODONTOPYGIDAE			
<i>Chaleponcus digitatus</i>	Kruger National Park	25°05'S. 31°42'E	October - February 1993-4
<i>Chaleponcus hangklip</i>	Moreletta, Pretoria	25°40'S. 28°12'E	December 1994
<i>Chaleponcus limbatus</i>	Marondera, Zimbabwe	18°10'S. 31°30'E	January - February 1993
<i>Chaleponcus oschei.</i>	Marondera, Zimbabwe	18°10'S. 31°30'E	January - February 1993
<i>Chaleponcus sp.</i>	Hwange, Zimbabwe	18°35'S. 26°20'E	January - February 1993
<i>Spinotarsus fiedleri complicans</i>	Pretoria	25°50'S. 28°12'E	October 1995
<i>Spinotarsus skukuzius</i>	Kruger National Park	25°05'S. 31°42'E	October - February 1993-4
<i>Spinotarsus sp.1</i>	Marondera, Zimbabwe	18°10'S. 31°30'E	January - February 1993
<i>Spinotarsus sp.2</i>	Big Bend, Swaziland	27°10'S. 31°40'E	October 1992
<i>Spinotarsus sp.3</i>	Kruger National Park	25°05'S. 31°42'E	October-November 1992

APPENDIX C

PRINCIPLES OF LIQUID SCINTILLATION COUNTING

Liquid scintillation counting is defined by the incorporation of a radiolabelled sample into homogeneous distribution with a liquid chemical medium (scintillation cocktail) that converts the kinetic energy of nuclear emissions into emitted photons (Faires and Boswell 1981).

When an imbalance exists between the number of protons and neutrons in a nucleus, the element becomes unstable and a rearrangement takes place. During these rearrangements, particles are given off, representing a decay or a radioactive event, and energy is released in the form of radiation.

Tritium is a radioactive isotope of hydrogen with an excess of neutrons in its nucleus. The decay results in the emission of two particles, an electron given the name beta particle because it originates in the nucleus, and a neutrino. These two particles are released simultaneously and carry the decay energy of 18,6 keV from the nucleus. Because the decay energy is shared between the neutrino and the beta particle, the beta particle of tritium can theoretically possess any energy between 0 and 18.6 keV, and a maximum energy of 18.6 keV.

The energy is kinetic and is dissipated by collisions in the medium in which it is released. The energy is absorbed by the medium in three forms: heat, ionisation and excitation of the molecules of the solution. Liquid scintillation counting measures the excitation of the solution's molecules.

To ensure the efficient transfer of energy between the beta particle and the solution, the sample carrying the isotope is placed in a scintillation cocktail, which is a solvent for the sample material. Excited solvent molecules are not readily recognised and therefore the scintillation solution (cocktail) contains a solute which is a fluor. Excited solvent molecules pass energy to each other and to the solute. When an excited solvent molecule passes its energy to a solute molecule, the orbital cloud of the solute is

disturbed and is raised to a state of excitation. As the excited orbital electrons of the solute molecule return to the ground state, a radiation results. In this case it is in the form of a photon of light. Thus a single beta particle will manifest its presence by colliding with solvent molecules, resulting in the excitation of many fluor molecules.

The total number of photons from the excited fluor molecules constitutes the scintillation. The intensity of light is proportional to the initial energy of the beta particle (which was between 0 and 18,6 keV). It is quantified by placing a vial containing the radiolabelled sample and the scintillation cocktail into a dark enclosure. Here photomultiplier tubes detect the scintillations and convert them into electrical pulses for counting.

The energy used for calculations of absorbed energy is an average energy based on the maximum energy and the atomic number of the nucleus. Again, this is an energy characteristic of a particular radionuclide, and each radionuclide can be uniquely identified by its unquenched average energy.

Because photomultiplier tubes produce small electrical pulses ("noise pulses") even when no light is present, it is necessary to quantify background counts in order to obtain accurate representations of nuclear emissions.

Several factors can reduce the counting efficiency of the solute-solvent system. These include photon quenching (incomplete transfer of beta particle energy to solvent molecules), chemical quenching (energy losses in the transfer from solvent to solute) and optical quenching (the attenuation of photons produced in the solution). The total effect is called quenching and the result is energy loss in the liquid scintillation solution. As a result, the energy spectrum of the radionuclide appears to shift toward lower energies. Counting efficiency is therefore dependent on the degree of quenching and thus on the nature of the sample, the scintillator used and the preparation technique.

It is therefore essential to monitor the counting efficiency in each sample by comparisons with standards or other samples. In modern scintillation counters, the counting efficiency is determined for each sample and the detected counts are converted to disintegrations to correct for quenching effects.

APPENDIX D

SPERM COMPETITION IN THE ODONTOPYGIDAE: SOME PRELIMINARY RESULTS

D.1 Preamble

The experimental work on *Spinotarsus* sp.1 was conducted close to the inception of this study and does not conform to the experimental protocols for the iterative experiments on *A. uncinatus* and *P. diplodontus*. Subsequent work on *Chaleponcus digitatus* is also incomplete due to difficulties encountered in collecting sufficient animals due to drought. It was not possible to compare these data with those from the Spirostreptidae and Harpagophoridae due to the lack of controls (dpm counts from females mated with unlabelled males) and small sample sizes (acceptance of the null hypothesis could signify type II error). These results do nevertheless shed some light on the timing of insemination and patterns of ejaculate transfer and storage in the Odontopygidae.

D.2 Methods

Spinotarsus sp. 1 (body mass: male: 1.9 ± 0.2 g, $n = 71$; female: 2.5 ± 0.4 g, $n = 50$) was collected from Marondera, Zimbabwe in January and February 1993. *Chaleponcus digitatus* (body mass: male: 1.9 ± 0.3 g, $n = 36$; female: 2.7 ± 0.3 g, $n = 20$) was collected from the Kruger National Park in January and February 1994 (see Appendix B for collection details). Animals were separated by sex and housed in glass aquaria (30 x 30 x 30 cm) lined with vermiculite, and fed fresh fruit and vegetables *ad libitum*. Experiments were conducted at the Universities of Zimbabwe, Harare (*Spinotarsus* sp.1) and Pretoria (*C. digitatus*).

Mating sequences in which females were mated sequentially with labelled (L) and unlabelled (UL) males were conducted. Labelled males were injected with 30 μ l tritiated [methyl- 3 H]thymidine, administered with a Hamilton syringe between body segments 10 and 11 (as for *A. uncinatus* and *P. diplodontus*, Chapter 4). Unlabelled males were injected with an equivalent volume of insect saline. Injections neither

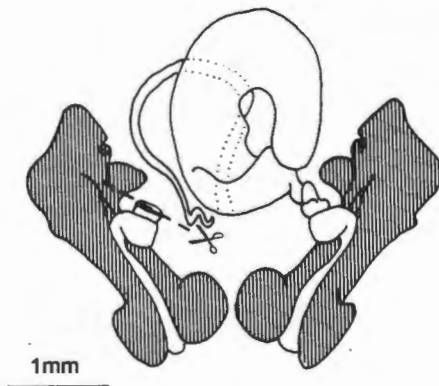


FIGURE D.1 Telopodite manipulation as performed on the gonopods of male *Spinotarsus sp.1*. The right telopodite was severed close to its point of emergence from the coxite. □ = telopodite; ▣ = coxite.

affected the copulatory behaviour of the males nor their copulation durations (copulation duration injected vs uninjected: *C. digitatus*: Mann-Whitney $U = 422$, $P = 0.289$, $n = 21$, 15 , $df = 34$; *Spinotarsus sp.1*: Mann-Whitney $U = 194.5$, $P = 0.3381$, $n = 13$, 20 , $df = 31$; mean copulation durations: *C. digitatus* = 38.7 ± 25.3 mins, $n = 20$; *Spinotarsus sp.1* = 25.05 ± 9.07 mins, $n = 91$).

C. digitatus females were used in both single (L only) and double (L-UL (0); UL-L (0)) mating sequences, but impact of a delay between or following matings was not investigated. *Spinotarsus sp.1* females were used in L only, L-UL (24) and UL-L (24) sequences, as well as in telopodite manipulation sequences (UL-L(M) (24) and L-UL(M) (24)). The manipulation was performed by severing the right telopodite close to its point of emergence from the coxite (Fig. D.1). Mating sequences without a delay between matings were not conducted for this species.

Immediately following mating, females were sacrificed in ethyl acetate killing jars and their vulvae were processed for scintillation counting (see Chapter 4). Vulvae were not divided into top and bottom components. Dpm were counted using either a Packard Scintillator 299 (*Spinotarsus sp.1*) or a JEOL840 Scintillation counter (*C. digitatus*). Both machines were programmed to count all samples three times and to automatically subtract the background count.

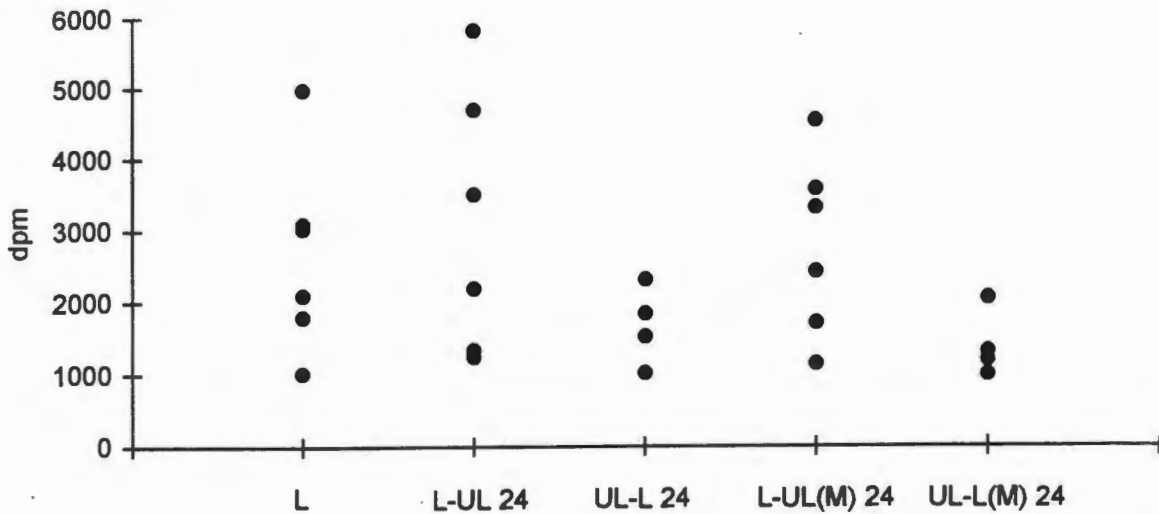


FIGURE D.2 *Spinotarsus* sp.1 dpm values for basic sequences and those involving the telopodite manipulation (dpm from left and right vulvae together). Data points (•) represent the dpm counts for individual females.

D.3 Results and discussion

Spinotarsus sp.1

Dpm did not differ between the L, L-UL(24) and UL-L(24) sequences of *Spinotarsus* sp.1 (Kruskal Wallis test: $H = 2.23$; $df = 2$; $P = 0.328$). If it is assumed that males inseminate equivalent ejaculate volumes initially, then female mediation of competition in the 24 hours post insemination (as in *A. uncinatus*; see Chapter 6) can be excluded.

Because female mating histories were unknown, mating sequences in which the contribution of first males were counted potentially include counts for both virgins and non-virgins. Mating sequences quantifying the contribution of second males only reflect counts of the contribution to non-virgins. A consequence of first male precedence (i.e. $V_2 < 0.5$) from experimental protocols involving females with unknown mating histories would be low dpm counts for non-virgins and high counts for virgins. This would be reflected in greater variances of dpm counts for the L only and L-UL sequences (potentially both virgins and non-virgins), and lower for the UL-L sequences (non-virgins only). This is supported by the data (Fig D.2): all mating sequences produced low dpm counts and only the sequences in which females may have been

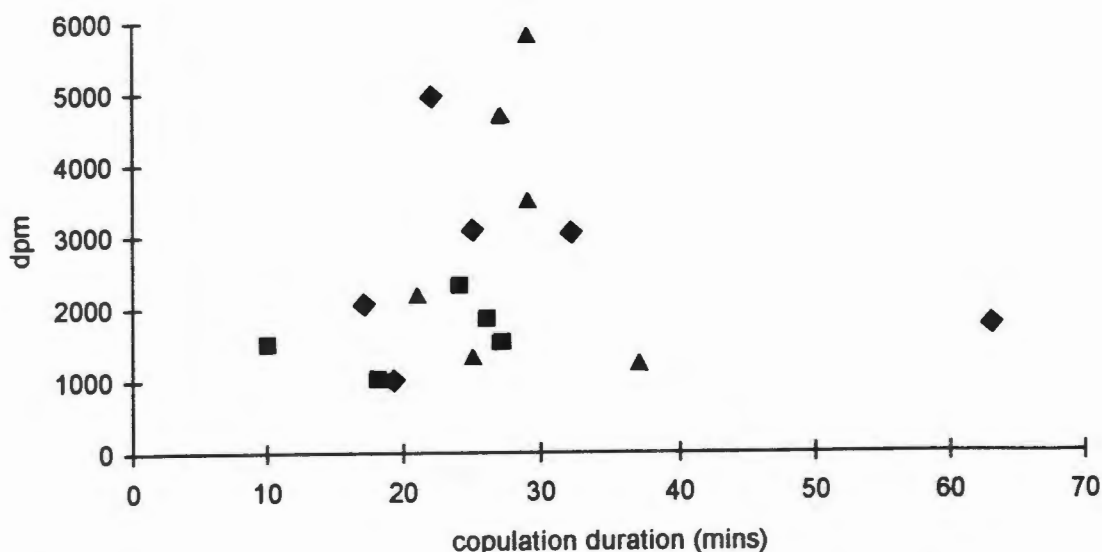


FIGURE D.3 Dpm counts vs copulation duration for *Spinotarsus* sp.1. Dpm values from double mating sequences are plotted against the copulation duration of labelled males (i.e. Male 1 from the L-UL sequence and Male 2 from the UL-L sequence. \blacklozenge = L; \blacksquare = UL-L; \blacktriangle = L-UL). Regression equation: $\text{Dpm} = 5.48 \text{ mins} + 2374$; $r = 0.04$; $n = 17$; $P = 0.87$.

virgins produced some high dpm counts. (The L and L-UL sequences (in which the first male's ejaculate was labelled) have higher variances (due to several high data points) than the UL-L mating sequence).

As above, the implications of first male precedence in ejaculate volume (i.e. V_2) for P_2 depend on both sperm distribution patterns within ejaculates and ejaculate distribution patterns within the sperm stores. All things being equal, $P_2 < 0.5$. The lack of a significant result in these data could be the consequence of type II error (Zar 1984).

Spinotarsus sp. 1 dpm counts were plotted against copulation duration. Due to the absence of naturally occurring short copulations, patterns of ejaculate transfer over the first phase of copulation are not clear (Fig. D.3). The possibility that first male precedence operates also confounds the interpretation of this figure: the high dpm values around 30 mins do not necessarily reflect an increase in ejaculate volume transferred with time. They may simply be data points corresponding to virgin females. There is no indication that males adjust copulation duration in response to mating order, because copulation durations of first and second males did not differ (Wilcoxon test: $T = 32.5$; $P = 1$; $n = 12$).

Telopodite manipulation sequences revealed no left-right differences for either manipulation sequence (L-UL(M) 24 left vs right vulvae Wilcoxon test: $T = 12$; $n = 6$; $P = 0.834$; UL-L(M) 24 left vs right vulvae Wilcoxon test: $T = 2$; $n = 4$; $P = 0.361$) indicating that the telopodite does not play a role in either ejaculate displacement or placement. This finding is consistent with other results: (1) basic mating sequences indicate that second males do not affect prior ejaculates and (2) the distal telopodite does not make contact with the coxite (where the coxite plays a role in ejaculate movement, ejaculates must be transferred from the coxite to the telopodite). These data also support the above interpretation of first male precedence (Fig. D.2). Copulation durations of males with telopodite manipulations did not differ from those with intact gonopods (Mann-Whitney $U = 1025$; $n = 40$; 10 ; $P = 0.91$).

Assuming first male precedence occurs in *Spinotarsus* sp.1, then this may be a consequence of large ejaculate sizes rather than small vulvae: single matings with labelled males suggest that *Spinotarsus* sp.1 males inseminate ejaculate volumes that are an order of magnitude larger than any other species examined (including *C. digitatus* which is of similar body size and was injected with an equivalent volume of isotope, see below). This increase in ejaculate size corresponds to an increase in vulval size. Those of *Spinotarsus* sp.1 are larger than any other Odontopygidae species examined (and seemingly disproportionately so, relative to body size).¹

First male precedence is also consistent with the manner in which *Spinotarsus* sp.1. males mount females. Once a male makes contact with a female, he rapidly coils around her posterior end and moves, in this coiled position, up her body. This species is unusual in that contact with females always results in mounting which always results in copulation. In all other Spirostreptida species that have been observed, females seem to be able to prevent copulation even after males are coiled around their bodies.

Further evidence for first male precedence is that copulation duration is shorter in this species than in any other species examined in this study. Where first male precedence occurs, males would accrue no benefits from mate guarding. Given that insemination is completed within 30 minutes and is probably early, a "strategy" to maximise

¹ *Spinotarsus* sp.1 vulvae are similar in size to those of *A. uncinatus* (Spirostreptidae), a much larger animal (*Spinotarsus* sp.1 mean mass = 2.5 ± 0.4 g, $n = 45$; *Alloporus uncinatus* mean mass = 9.3 ± 5.9 g, $n = 295$).

TABLE D.1 Dpm (disintegrations per minute) counts for *C. digitatus* and *Spinotarsus* sp. 1 mating sequences.

Species	Mating sequence	Mean dpm \pm s.d.	n
<i>C. digitatus</i>	L	616.4 \pm 136.91	4
"	L-UL (0)	445.16 \pm 300.6	8
"	UL-L (0)	477.12 \pm 292.67	8
<i>Spinotarsus</i> sp. 1	L	2655.44 \pm 1256	6
"	L-UL (24)	3120 \pm 1708	6
"	UL-L (24)	1586 \pm 464	5
"	L-UL(M) left	1366.53 \pm 609.98	6
"	L-UL(M) right	1403.52 \pm 663.21	6
"	UL-L(M) left	600.16 \pm 367.91	4
"	UL-L(M) right	774.23 \pm 110.99	4

reproductive success would be one in which males copulate rapidly with as many females as possible, in the hope of finding virgins before other males do. In the Sierra dome spider *Linyphia litigiosa*, first male precedence operates and males vary their copulatory behaviour in response to female mating histories (they are more aggressive when competing for virgins, Watson 1990; 1991). Because female mating histories were unknown, it is not clear whether male *Spinotarsus* sp.1 distinguish virgins from non-virgins similarly, and adjust copulation durations accordingly.

Chaleponcus digitatus

Dpm were also not significantly different for the *C. digitatus* L, L-UL(0) and UL-L(0) mating sequences (Kruskal Wallis test: $H = 2.03$; $df = 2$; $P = 0.365$; Table D.1). If it is assumed that ejaculates were evenly distributed with the label, it can be concluded that both males contribute equivalent volumes of ejaculate to the vulvae ($V_2 = \pm 0.5$), and the second ejaculate is not volumetrically constrained by the first. If ejaculates are evenly distributed with sperm and a raffle ensues (i.e. sperm are used for fertilisation in proportion to their numbers), second males also have a P_2 of ± 0.5 .

Dpm were plotted against copulation duration to elucidate patterns of ejaculate transfer. Data suggest early insemination and the transfer of greater volumes of ejaculate than typically remain in the vulvae following copulation, similar to both *A. uncinatus* and *P. diploodontus* (Chapter 4) (Fig D.4). There were no differences

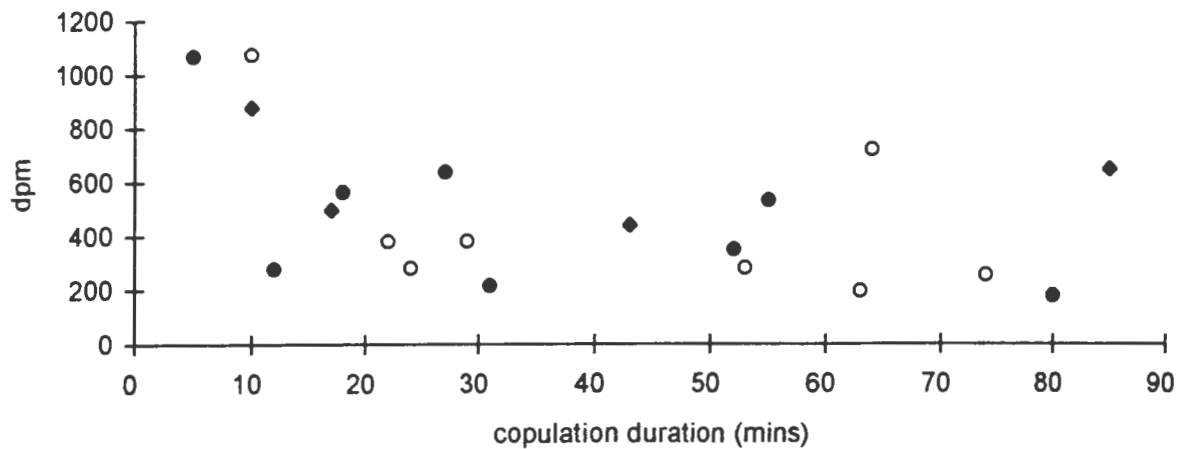


FIGURE D.4 Disintegrations per minute (dpm) counts vs copulation duration (mins) for *C. digitatus*. Dpm values from double mating sequences are plotted against the copulation duration of labelled males (i.e. Male 1 from the L-UL sequence and Male 2 from the UL-L sequence). ◆ = L; ● = UL-L; ○ = L-UL. Regression equation: $Dpm = 672.5 - 4.65 \text{ mins}$; $r = 0.43$; $n = 20$; $P = 0.06$.

between the copulation duration of first and second males (Wilcoxon test: $T = 41$; $n = 16$; $P = 0.293$).

D.4 Summary

Direct sperm displacement is not indicated in either Odontopygidae species, and in *Spinotarsus* sp.1 the telopodite is not implicated in ejaculate transfer either. These findings are consistent with Odontopygidae genital morphology: (a) it is unlikely that the telopodite has direct access to the spermathecae because bursae are located deeply within the vulval sacs and are partly obscured by vulval tissue (Chapter 3), and (b) distal telopodites do not make contact with the coxite (discussed above and see Chapter 2).

Interestingly, patterns of ejaculate transfer were not conserved within the family: in *C. digitatus* $V_2 = \pm 0.5$; in *Spinotarsus* sp.1 first male precedence may operate ($V_2 < 0.5$). Differences between these species in genital morphology may be related to the mechanisms of competition.

APPENDIX E

PREVIOUS PUBLICATION OF THIS WORK

Some of the data presented in this thesis has been published previously. Details of these publications follow. All the papers are co-authored by my principal supervisor Dr S.R. Telford, who conceptualised the original design of the experimental work. Dr B.R. Tibbles is the third author of Barnett *et al.* 1995 due to his involvement in early methodological investigation.

1. Barnett, M. and Telford, S.R. 1994. The timing of insemination and its implications for sperm competition in a millipede with prolonged copulation. *Anim. Behav.* 48:482-484.

This short communication investigated the timing of insemination in *A. uncinatus* (Spirostreptidae) using tritiated thymidine to label ejaculates. The data published in this paper form part of Chapter 4 of this thesis, in which they are combined with those from a similar but more substantial experiment on *P. diplodontus* (Harpagophoridae).

2. Barnett, M., Telford, S.R. and Tibbles, B.J. 1995. Female mediation of sperm competition in the millipede *Alloporus uncinatus* (Diplopoda: Spirostreptidae). *Behav. Ecol. Sociobiol.* 36: 413 - 419.

In this paper radiolabelling techniques were combined with double mating experiments to investigate sperm precedence in *A. uncinatus* (Spirostreptidae). Some of the interpretation in the original paper, including the inference of P_2 from ejaculate volumes, was based on incorrect assumptions about female vulval morphology. With a more thorough appreciation of female reproductive morphology (Chapter 3), this data is reinterpreted in Chapter 6. A copy of this paper is attached for reference purposes.

3. Barnett, M. and Telford, S.R. 1996. Sperm competition and the evolution of millipede genitalia. *Mémoires du Muséum national d'Histoire naturelle*. 169: 331 - 339.

This paper was published in the conference proceedings of the 9th International conference of Myriapodology, held in Paris in 1993. The data reported here constitute part of Chapters 2 and 3 of this thesis. The abstract of the paper follows:

Gonopods of eight species of Spirostreptida millipedes were examined using light and scanning electron microscopy, and the sequence of events representative of the movement of the gonopods within the spermathecae demonstrated through the dissection of freeze-dried copula pairs and simulations using scale models. Gonopods bear devices that may function in sperm displacement, including flagellae with ridges and overlapping plates, scoops and regions of pitted spines. These are oriented correctly so as to facilitate sperm removal and are accommodated within the spermathecae of the females. This morphological evidence, coupled with Spirostreptida physiology and behaviour, indicates that sperm competition may have played a major role in shaping gonopod morphology.

M. Barnett · S.R. Telford · B.J. Tibbles

Female mediation of sperm competition in the millipede *Alloporus uncinatus* (Diplopoda: Spirostreptidae)

Received: 28 March 1994/Accepted after revision: 29 January 1995

Abstract The tropical spirostreptid millipede *Alloporus uncinatus* has a polygynandrous mating pattern that is apparently shaped by sperm competition (Telford and Dangerfield 1990, 1993a). In the present study radioisotopic labelling of ejaculates was used to quantify the temporal effects of double mating sequences on sperm precedence patterns. Ejaculates of successive males mix completely within the sperm storage organs and are concentrated close to the site of fertilisation, the spermathecal-oviduct junction. When matings follow each other immediately (experiment 1), equal volumes of the ejaculates of each male are stored within the spermathecae, and both males have equal probability of paternity. Where matings are separated by a 24-h delay (experiment 2), the volumetric contribution of the first male is reduced by 54.8% and last male precedence operates. Although the distal ends of the gonopods play a primary role in the redistribution of ejaculates (Barnett and Telford 1994), by using a single mating sequence we show that they are not responsible for the reduction in ejaculate volume. The most likely explanation is absorption and/or ejection of the first male's ejaculate by the female. Genital functional morphology is used to reconstruct the mechanism of ejaculate distribution.

Key words Sperm competition · Female choice · Millipede

Introduction

Where females are multiply mated and sperm are stored, the opportunity exists for sperm of different males to compete directly for the fertilisation of ova (Parker 1970a). In invertebrates with internal fertilisation, the arenas for sperm competition are the sperm storage organs of the female reproductive tract, the spermathecae. Here competition between rival ejaculates results in selection for behavioural, morphological and physiological traits of both males and females to enhance and prevent preemption of previous ejaculates (Parker 1970a; Smith 1984; Birkhead and Møller 1993).

Male adaptations to sperm competition are widely recognised and include mate guarding (e.g. Parker 1974; Waage 1979a; Sillén-Tullberg 1981; Telford and Dangerfield 1990), removal of rival ejaculates (e.g. Waage 1979b) and stratification of ejaculates such that they are displaced to regions of the spermathecae where fertilisation is less likely to occur (Siva-Jothy 1988; Walker 1980). Female adaptations are less obvious and have often been considered to be of secondary importance (Knowlton and Greenwell 1984; Birkhead and Møller 1993). Females can store sperm for long periods and in the field successive matings are not always immediate. Where copulation does result in insemination, insemination does not always result in fertilisation (Eberhard 1985, 1991), and females may exercise ultimate control over offspring paternity (cryptic female choice, *sensu* Thornhill 1983). Processes of postcopulatory (but prefertilisation) female choice within the female reproductive tract include selective utilisation, expulsion and absorption of ejaculates (Villavaso 1975; Gwynne 1984; Birkhead and Møller 1993).

The double mating experimental paradigm employed in studies of sperm competition typically allows the second male to copulate with a female directly after the first (Parker 1970b; Smith 1984), and effectively prevents females from influencing the

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storage and use of the first ejaculate before the second male can also influence its fate (Ward 1993). It is therefore not surprising that the literature contains many documented examples of flushing or direct removal of sperm by males (Smith 1984; Birkhead and Hunter 1990) but relatively few examples of female control of paternity and the likely mechanisms involved (Knowlton and Greenwell 1984; but see Walker 1980; Ward 1993).

Millipedes are polygynandrous, females store sperm and fertilisation is delayed (Hopkin and Read 1992). The reproductive openings, gonopores, of millipedes occur on their second body segment. In females the gonopores lead to paired spermathecae that give rise distally to oviducts (Fig. 1). In males they terminate in non-functional penes. In addition to these, males bear a pair of accessory genitalia, gonopods, on their seventh body segment (Fig. 2) that function as sperm displacement devices (for removal see Barnett et al. 1993; for redistribution see Barnett and Telford 1994). The gonopods are usually held within the body of the male, but during copulation are protruded and translocate sperm from the penes to the spermathecae.

Copulation in the tropical millipede *Alloporus uncinatus* is initiated when a male walks up the back of a female, and coils himself around her such that their ventral sides are contiguous and their mouthparts are engaged (Fig. 3). The gonopods are first brought in contact with the penes and then inserted into the spermathecae, where they remain for the duration of copulation. Copulation lasts on average 98 min (Barnett and Telford 1994), is male mediated (Telford and Dangerfield 1993b, 1994) and has been interpreted as a form of mate guarding (Telford and Dangerfield 1990). Insemination is almost instantaneous and males have been shown to redistribute their own ejaculates closer to the site of fertilisation during copulation (Barnett and Telford 1994).

This is the first study to investigate sperm competition in a myriapod. We use radioactive labelling techniques to examine sperm precedence patterns in *A. uncinatus*. Specifically we test (a) whether sperm precedence patterns are a function of the temporal delay between successive matings and (b) whether they are determined by male or female controlled processes. Finally we reconstruct the sequence of gonopod movement within the spermathecae and explain the observed precedence patterns in the context of genital functional morphology.

Materials and methods

Animal maintenance and collection

Animals were collected from a site adjacent to the Mazowe dam, Zimbabwe (17°30'S, 30° 57'E) in January 1993. They were housed in unisex groups of up to 30 individuals in vermiculite-lined glass

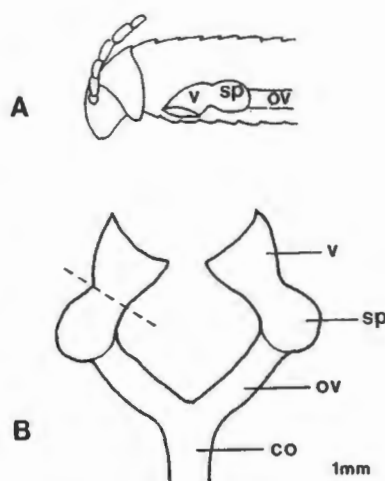


Fig. 1A,B The spermathecae of *Alloporus uncinatus* (B) and their *in situ* orientation in the body of a female (A) (not drawn to scale). The broken line indicates the point of division of the spermatheca into top and bottom components (v vulva; sp spermatheca, ov oviduct, co common oviduct)

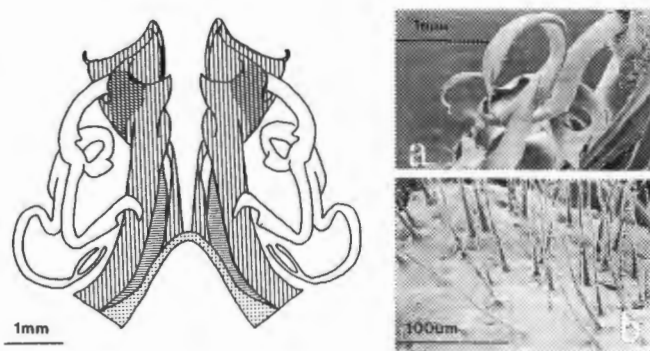


Fig. 2 Schematic representation of the gonopods of *A. uncinatus*: coxite (▨), region of spines (▩), telopodite (□), sternite (▧), gonocoel (▦), and scanning electron micrographs of the left telopodite arm (plate a) and spines on the distal coxite of the right gonopod (plate b)

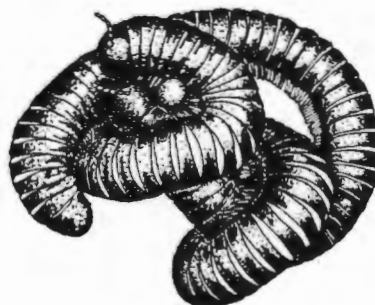


Fig. 3 A copula pair of *A. uncinatus* with the male coiled around the body of the female. The everted collar of the gonopods is visible between the heads of the two animals

aquaria (50 × 30 × 50 cm), and food in the form of fresh vegetables was provided *ad libitum*. They were maintained under ambient laboratory conditions (25°C; 75% RH) throughout the month-long study period.

Ejaculate labelling

Traditionally, studies of invertebrate sperm competition have quantified sperm precedence patterns by using genetic markers or irradiation techniques to determine the proportion of an egg clutch sired by the last male to mate (Parker 1970b). However, millipedes do not readily oviposit under laboratory conditions, and the determination of sperm precedence patterns through a measure of paternity was not possible. Instead precedence patterns were estimated by comparing the volumes of stored sperm in females. By using tritiated thymidine to label the ejaculates of males (*sensu* Barnett and Telford 1994) (a) the relative volumes of ejaculates of different males and (b) their distribution within the spermathecae were estimated. An important advantage of this approach is that it elucidates the mechanism of sperm displacement (Waage 1986).

Mating sequences

To determine if sperm displacement occurs in *A. uncinatus*, serial matings with males having unlabelled sperm and males whose sperm had been labelled with tritiated thymidine were conducted. "Labelled" males (L) were injected on their ventral surfaces between body segments 10 and 11 with 50 μ l [*methyl*- 3 H]thymidine (85 Ci/mmol, Amersham, UK), using a 50 μ l Hamilton syringe. To control for possible side effects of the injection, "unlabelled" males (UL) were injected with an equivalent volume of sterile, distilled water. All injections took place 24 h before males were used in mating sequences. It was assumed that all ejaculates were homogeneously labelled with the isotope and evenly distributed with sperm (see Barnett and Telford 1994). The absence of a relationship between male body mass and dpm (disintegrations per minute) indicated no difference in the intensity of radioisotope labelling of ejaculates of males of different sizes (Pearson's correlation coefficient: $r = 0.18$, $P = 0.53$, $n = 14$, two-tailed test).

Initially two series of experiments were conducted, each comprising two mating sequences. In the first sequence each female was mated first with a labelled male and then with an unlabelled male (L-UL). These results were used to quantify the ejaculate contribution of the first male. In the second sequence, the order was reversed and each female was mated first with an unlabelled and then with a labelled male (UL-L), thus quantifying the contribution of the second male. In the first experiment, matings followed each other immediately and in the second, the delay between matings was 24 h.

Copula pairs were generated by placing up to five animals of each sex in glass aquaria similar to those in which they were housed. Upon their formation, pairs were translocated from the aquaria to plastic beakers (13 cm diameter) and the duration of each mating was recorded. Both animals appeared to be unaffected by the move which was necessary to prevent interference from single males (personal observations, and see Telford and Dangerfield 1993a,b).

Scintillation counting

Immediately following the termination of their second mating, females were placed in an ethyl-acetate killing jar. Their spermathecae were dissected out and each spermatheca was divided into top and bottom components (Fig. 1). These were placed separately into 4-ml pony vials and vortexed with 0.1 ml concentrated HCl to disintegrate the tissue (and thus prevent differential quenching). The acid was neutralised with 0.1 ml 5 M NaOH prior to the addition of 3.5 ml scintillation cocktail (Scintillator 299, Packard). Disintegrations per minute were determined in a Packard 1600 scintillation counter (low count reject = 0, dpm multiplier = 1) and the mean disintegration rate (± 1 SE) for three 10-min determinations per vial was recorded. The tritium counting protocol automatically subtracted the background count (50.6 ± 1.95 dpm, $n = 3$) from all

measures of disintegrations per minute. Disintegration rate of spermathecae from females who had mated once with unlabelled (UL) males were determined as a control. These values were consistent with values for the background count (52.5 ± 60.13 dpm).

Scanning electron microscopy (SEM)

Males ($n = 6$) were placed in ethyl-acetate killing jars and their gonopods were removed, thoroughly rinsed in physiological saline and fixed in 2.5% glutaraldehyde (pH 7.4, phosphate-buffered saline) at 4°C for 24 h. Secondary fixation in osmium tetroxide (2%) was followed by dehydration through a graded alcohol series (30% through absolute ethanol) and critical point drying. Samples were mounted on specimen stubs, sputter-coated with gold-palladium and examined under a Cambridge S200 scanning electron microscope.

Simulation of gonopod action

Copula pairs were separated by carefully uncoiling the male from around the body of the female. When pairs were separated but their mouthparts were allowed to remain engaged, the male continued to retract and release the telopodites of his gonopods (see Fig. 2 for an explanation of terminology). By observing these telopodite movements and using SEM and dissections of copula pairs that had been frozen in liquid nitrogen, the sequence of gonopod movement during copulation was reconstructed (and see Barnett et al. 1993 for a detailed description of the methodology).

Statistical analysis

Statistical analyses were performed using Minitab version 7.2 (Minitab, Inc. 1989) software. Disintegration counts did not conform to the assumptions of parametric tests and samples were too small for the data to be easily transformed to normality, thus non-parametric statistical analyses were used (Siegel and Castellan 1988). Significance tests were two-tailed except for multiple comparisons between treatments (χ statistic, Siegel and Castellan 1988) for the Kruskal-Wallis one-way analysis of variance, which were one-tailed. The alpha level of acceptance for all tests was 5%. Two-tailed Pearson's correlation coefficients were used in analyses of covariation involving body mass, copulation duration and disintegration rate.

Results

Precedence patterns

When the second mating immediately followed the first (experiment 1), there was no significant difference in disintegration rate between the first and second mating sequence (L-UL vs. UL-L: two-tailed Mann-Whitney U -test, $W=64$, $P=0.71$, $df=14$, and see Table 1). A delay of 24 h between the first and second mating (experiment 2) yielded a significantly lower disintegration rate for the first mating sequence (L-UL vs. UL-L, $W=26$, $P=0.03$, $df = 11$, and see Fig. 4, Table 1). These results suggest that when there is no delay between matings females store an equivalent volume of both males' ejaculates, but when a 24-h delay is effected, there is a significant reduction in the contribution of the first male. This cannot be a consequence

Table 1 Mean (± 1 SE) disintegrations per minute (dpm) and the proportion of ejaculates placed at the bottom of the spermathecae for the two series of double mating experiments (*L* labelled male, *UL* unlabelled male)

Hours between matings	Mating sequence	Dpm (\pm SE)	<i>n</i>	% Dpm at bottom of spermathecae
0	L-UL:	1131.89 (\pm 462.14)	8	55.39% (\pm 4.58%)
	UL-L:	1317.59 (\pm 618.13)	8	54.27% (\pm 3.82%)
24	L-UL:	467.76 (\pm 312.48)	6	58.07% (\pm 4.04%)
	UL-L:	1012.00 (\pm 507.2)	5	59.33% (\pm 8.02%)

of second males mating longer when a 24-h. delay between matings was imposed, as copulation duration of second relative to first matings did not differ as a function of the interval between matings ($W = 240.5$, $P = 0.14$, $df = 25$). The reduction could therefore either be a consequence of displacement by the second male to mate, or a consequence of absorption or expulsion of part of the first ejaculate by the female during the 24-h delay.

To distinguish between these alternative explanations, additional matings were conducted in which females ($n = 7$) were mated once with a labelled male, and only dissected 24 h following this copulation. If the reduction in disintegration rate following the 24-h delay was a consequence of a female process, then the disintegration rate from these single matings would be equivalent to the disintegration rate from the L-UL mating sequence of experiment 2. If disintegration rate reductions were a consequence of a male process, then counts would be equivalent to the UL-L mating sequence of this experiment. Multiple comparisons of these results support the female process hypothesis (Kruskal-Wallis statistic: $H = 7.36$, $P = 0.03$, $df = 2$; L-UL vs. UL-L, $z = 7.57$, $P < 0.05$; L-UL vs. L, $z = 0.67$, $P > 0.05$; UL-L vs. L, $z = 6.91$, $P < 0.05$).

Irrespective of the time delay between matings, the proportion of disintegrations at the bottom of the spermathecae was the same for males in both mating sequences, suggesting complete sperm mixing (Table 1). Most (54.27–59.33%) of these ejaculates were placed at the bottom of the spermathecae, close to the oviduct junction (Fig. 1).

Gonopod functional morphology

The gonopods of *A. uncinatus* comprise three components, the coxite, the sternite and the telopodite (Fig. 2). As in all spirostreptid millipedes, the telopodite (Fig. 2, plate a) of the gonopods originates at the base and emerges from a sleeve formed by the coxite. The telopodite bifurcates shortly after emergence from the coxite and terminates in two branches. The thinner branch tapers at its distal end. The thicker bears the so-called sperm canal, and forks at its distal end. The distal end of the coxite bears a region of hair-like spines (Fig. 2, plate b).

Gonopod movement is effected through proximal retraction of the telopodite which enters the spermatheca during copulation (Fig. 2, and see Barnett et al. 1993). The resultant movement of the telopodite in *A. uncinatus* is described in Fig. 5. Initial retraction results in partial erection of the telopodite (steps *a*–*b*). The telopodite is then released, returning it to position *a*. This retraction-release motion between steps *a* and *b* is repeated about 20 times, and is then followed by full rapid retraction from step *a* through *e*, and release to *a* again. The entire sequence of telopodite partial and then full retraction is repeated throughout the duration of copulation. Sperm are transferred via the penes to the distal ends of the coxites which do not penetrate the spermathecae during copulation (Barnett et al. 1993). We believe the full retraction and release of the telopodite from *a* through *e* and back to *a* to be the mechanism by which sperm are translocated into the spermathecae. The redistribution of ejaculates from the top to the bottom of the spermathecae over the duration of copulation is probably a consequence of the continuous repetition of the *a*–*b* step of the cycle.

Covariation in disintegration rate

Ejaculate volume has previously been related to male body mass with larger males transferring larger ejaculate volumes (Rubenstein 1989; Berrigan and Locke 1991). However, in *A. uncinatus* there was no relationship between disintegration rate and male body mass ($r = 0.18$, $P = 0.53$, $n = 14$), indicating that the volume of ejaculate is not related to body size. Ejaculate volume has also been shown to increase with the duration of copulation (Rubenstein 1989). However, in *A. uncinatus* ejaculates are transferred at the beginning of

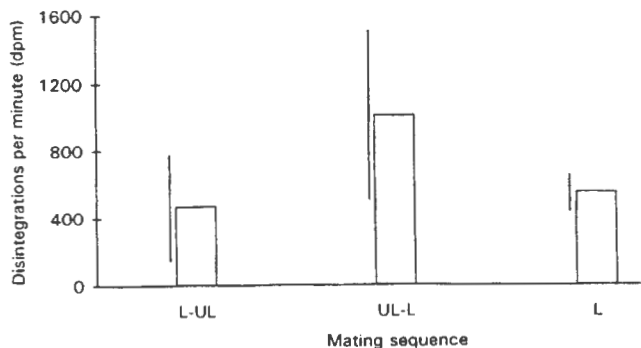
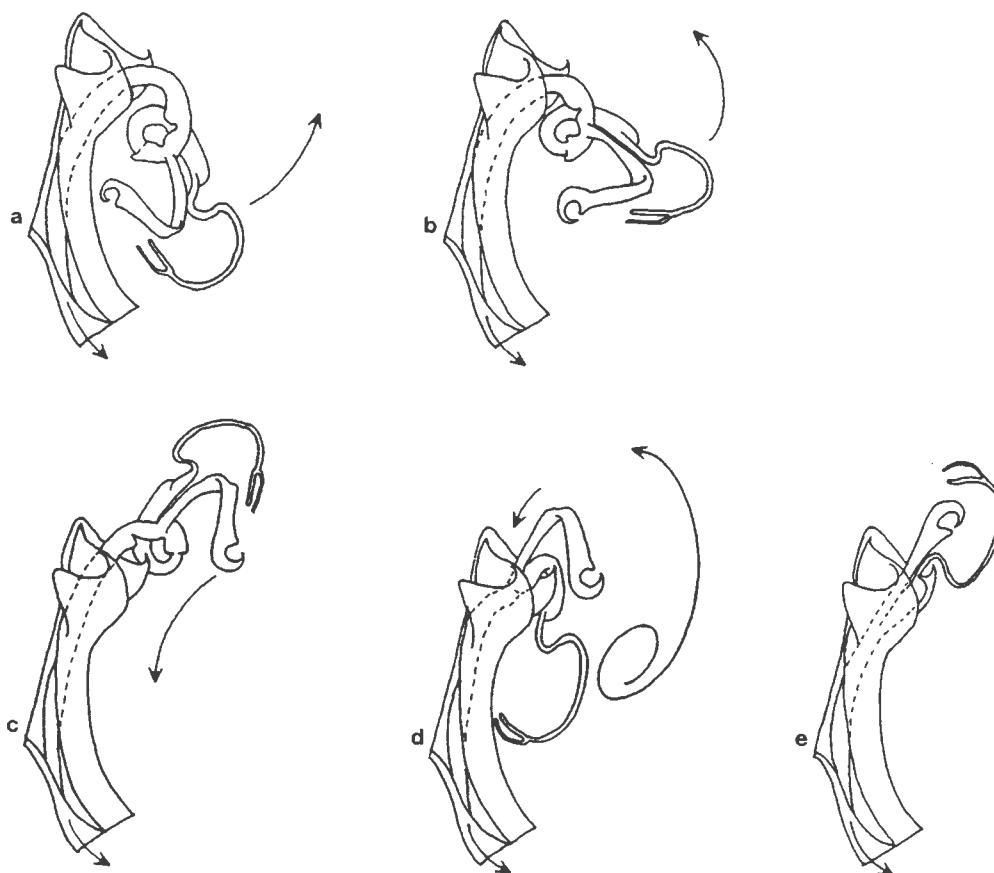


Fig. 4 Histograms (± 1 SE) of disintegrations per minute (dpm) for single and double (experiment 2) mating sequences (*UL* unlabelled, *L* labelled)

Fig. 5 Reconstruction of the telopodite retraction-release cycle of the right gonopod during copulation (for description of *a-c*, see text)



copulation (Barnett and Telford 1994) and predictably there was no relationship between disintegration rate and copulation duration ($r = 0.14$, $P = 0.63$, $n = 14$). If spermathecal size was positively correlated with female body mass and this size difference was related to spermathecal holding capacity, a relationship between female body mass and disintegration rate would be expected. No such relationship was evident ($r = 0.49$, $P = 0.07$, $n = 14$).

Discussion

Precedence patterns

Sperm precedence cannot usually be inferred from studies measuring sperm volumes because selective utilisation of sperm from different regions of the spermathecae may occur (McVey and Smittle 1984). However, if complete sperm mixing occurs prior to oviposition, it is likely that a raffle occurs and sperm are utilised for fertilisation in proportion to their numbers (Birkhead and Møller 1992, 1993). In this study, the proportion of ejaculate placed by successive males at the bottom of the spermathecae was the same, indicating that ejaculates mix completely (see below). Consequently, in *A. uncinatus*, the inference of P_2 (the

proportion of offspring sired by the last male to mate) from relative ejaculate volumes was possible. Female mating history prior to the experiments was not known and we do not take account of the possible presence of sperm from previous matings in our estimates of P_2 . The presence of such sperm would not affect the temporal trends in P_2 that we describe as the contribution of both males used in the double mating experiments would be devalued in direct proportion to the volume of sperm that was already present.

Previously several studies on sperm competition in arthropods that directly addressed the effect of temporal separation between successive matings on P_2 have shown a decrease in P_2 over time (e.g. Retnakaran 1974; Radwan 1991; Ward 1993). The volume of sperm displaced by the second male to mate has also been shown to decrease with a temporal delay between successive matings in the beetle, *Tenebrio molitor* (Gage 1992). These results were all attributed to the migration of sperm of the first male to regions of the female storage organs from where they could not easily be displaced. In *A. uncinatus* P_2 increased with the delay between matings. Neither male had a competitive advantage when matings followed each other immediately (experiment 1) and last-male precedence occurred when a 24-h delay was imposed (experiment 2).

The mechanism by which gonopods have been proposed to displace rival sperm is coupled to that of

insemination (Barnett et al. 1993) and, because insemination occurs early during copulation (Barnett and Telford 1994), it is probable that ejaculates mix within the spermathecae before a second male has the opportunity to selectively remove those of rival males. Under these conditions, selection for sperm removal would not be favoured because males would risk partial self-sperm removal (Parker G, cited in Birkhead and Møller 1993; Barnett and Telford 1994). Therefore, we believe the primary tactic of sperm competition in *A. uncinatus* to be sperm mixing. Although not an optimal means of ensuring paternity, the resultant dilution of the ejaculate of the first male to mate is effective in that it reduces the predicted fertilisation success of the first male by at least 50%.

The incidence of last-male precedence when a temporal delay between matings was imposed was not a consequence of ejaculate removal by the second male to mate. In those species of spirostreptid millipedes in which gonopods are believed to function in sperm removal (Barnett et al. 1993; Barnett and Telford, in press), the gonopods bear morphological structures analogous to sperm displacing devices of some insects (Waage 1982, 1986; Gage 1992). Although the gonopods of *A. uncinatus* redistribute ejaculates (Barnett and Telford 1994), they reveal no obvious morphological structures that could function in ejaculate removal. The structures on the distal ends of the coxite (Fig. 2, plate b) are hair-like in contrast to the short stout spines that have been proposed to function in displacement in the millipede *Orthoporus pyrhocephalus* (see Barnett et al. 1993 for a comparison). Further, the distal ends of the telopodite arms are smooth and taper (Fig. 2, plate a), bearing neither scoops (as in *O. pyrhocephalus*, Barnett et al. 1993) nor serrated plates (as in *Philoporatia dipodontus*, Barnett M and Telford SR, unpublished work).

Our results showed that the observed reduction in ejaculate volume was a consequence of a female-mediated process (see Fig. 4). Previous studies have shown females to manipulate ejaculates through selective utilisation (Birkhead and Møller 1993), expulsion (Villavaso 1975) and absorption (Gwynne 1984; Radwan and Witalinski 1991). No oviposition occurred during the experimental period and therefore loss of ejaculates through utilisation for fertilisation can be excluded. Therefore we believe the reduction in ejaculate volume to be a consequence of absorption and/or ejection of ejaculates by the female during the 24-h delay between matings (although our observations of females postcopula provided no evidence for ejaculate expulsion). Sperm mortality has also been suggested as a possible reason for a reduction in sperm number over time (Tsubaki and Yamagishi 1991), but is an unlikely explanation here as this would not have affected disintegration rate.

Male or female mediation of sperm competition?

These data illustrate how processes determining sperm precedence patterns can be both male- and female-mediated. In *A. uncinatus* males control the duration of copulation (Telford and Dangerfield 1990) and redistribute their own ejaculates closer to the bottom of the spermathecae (Barnett and Telford 1994). Walker (1980) predicted that sperm placed closest to the spermathecal-oviduct junction should achieve fertilisation precedence and because this junction occurs distally, sperm placed at the bottom of the spermathecae should enjoy precedence.

These events suggest that males dominate and females have little control over syn-copulatory events. However, where females have little choice between males or copulations are forced, it may still be feasible for females to have some physiological or anatomical control of paternity (Birkhead and Møller 1993). Our results show that females are responsible for ejaculate depletion during the temporal delay between matings, and this manipulation of ejaculates post copulation may afford females the ultimate control of offspring paternity.

Mechanism of ejaculate redistribution

We suggest that the movement of the telopodites during copulation are responsible for placement and mixing of ejaculates. Based on an understanding of gonopod functional morphology, the volumes of stored ejaculates and previous data suggesting ejaculate repositioning and self-sperm displacement, the following mechanism of ejaculate redistribution is proposed (Fig. 6):

1. Male 1 mounts a female (Fig. 3), everts his gonopods, and translocates his ejaculate from the penes via the distal end of the coxite into the spermathecae. Because the coxites do not penetrate the

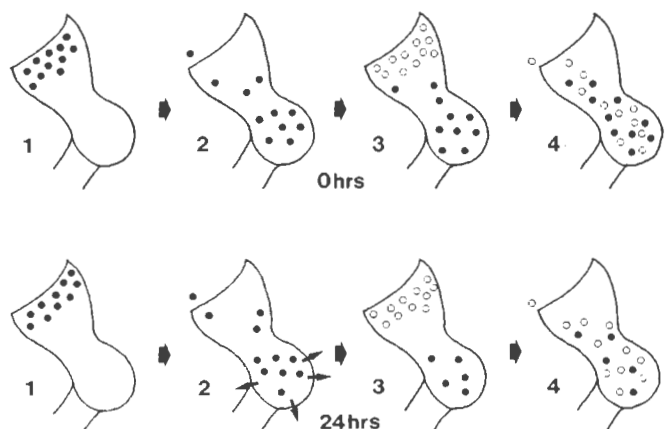


Fig. 6 Proposed mechanism of ejaculate distribution and redistribution within a spermatheca (● ejaculate of male 1, ○ ejaculate of male 2)

spermathecae. ejaculates are placed close to the entrances of the vulvae.

2. Male 1 then uses the distal ends of the telopodites (as in Fig. 5) to reposition the ejaculate close to the bottom of the spermathecae. This process is unlikely to be aided by gravitational forces as the spermathecae lie horizontal within the body of the female (Fig. 1). The redistribution causes some of the ejaculate to seep out of the spermathecal openings, resulting in only part of it remaining within the spermathecae.
3. The female is released, and is mounted by male 2 (either immediately, in which case the full complement of ejaculate remains, or after 24 h when the ejaculate volume of male 1 is reduced).
4. Male 2 translocates his ejaculate into the spermathecae, close to the vulval entrances. Again the telopodites are used to reposition the male's ejaculate close to the bottom, but because the first male's ejaculate is already there, repositioning results in mixing of the two ejaculates.

Acknowledgements Thanks to Solly and Lindy Levy for logistic support and John Loveridge for the use of his laboratory facilities at the University of Zimbabwe. Financial support from the Universities of Cape Town and Pretoria and the Foundation for Research and Development is gratefully acknowledged. Peter Henzi and Mandy Dyson made useful comments on an earlier version of the manuscript. We would also like to thank the three referees whose comments improved the final version of this manuscript.

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Communicated by M.A. Elgar

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