Spermatozoal ultrastructure of the hermit crab *Loxopagurus loxochelis* (Moreira, 1901) (Decapoda: Anomura: Diogenidae) from the southwestern Atlantic

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Abstract.—The spermatozoal morphology of *Loxopagurus loxochelis* exhibits characters shared by other paguroids: complex concentrically zoned acrosome vesicle, capped by an electron-dense operculum and posteriorly penetrated by a large perforatorial chamber, and three microtubular arms emanating from the cytoplasm. The general sperm cell dimensions and arrangement of the acrosome in *L. loxochelis* are similar to those previously described for three species of *Calcinus*. However, *Loxopagurus* lacks a defining autapomorphy of this genus: splitting of the anterior end of the perforatorial chamber into many obvious fingers. The only unique character observed in the sperm of *Loxopagurus* is the presence, in the cytoplasm, of a bulge filled with small tubules on the side of the acrosome. This structure could be an artifact or possibly a parasitic bacterial infection. In conclusion, *L. loxochelis* sperm morphology does not demonstrate any clear autapomorphies, but seems to separate from the other genera in the Diogenidae by the absence of their defining sperm characters (e.g. anterior perforatorial fingers and microvillar projections). It appears most similar to the genus *Calcinus*, and more superficially *Strigopagurus*, although its spermatophore morphology seems to indicate a closer relationship with *Clibanarius*. The combination of spermatophore and spermatozoal characters clearly places it in the Diogenidae, and supports its monotypic status.

INTRODUCTION

The anomuran crustaceans include a wide variety of forms ranging from the eye-catching hermit crabs to commercially valued species such as the king crabs. Some anomuran species are semiterrestrial, or freshwater, but

most are marine, inhabiting a broad bathymetric range, from littoral to deep waters. According to McLaughlin (1983), McLaughlin & Holthuis (1985), and Martin & Davis (2001), the Anomura comprise the superfamilies Lomisoidea, Galatheoidea, Hippoidea, and Paguroidea. The superfamily Paguroidea, to which the familiar hermit crabs and king crabs belong, includes the Coenobitidae, Diogenidae, Paguridae, Parapaguridae, Pylojacquesidae, Pylochelidae and Lithodidae (McLaughlin & Lemaitre, 2001; McLaughlin, 2003). The anomuran family Diogenidae is considered an ancient monophyletic group (Forest, 1995) and is a morphologically diverse taxon currently encompassing 19 genera (McLaughlin, 2003). In Argentinian waters, three families, six genera and ten species of “hermit crabs” have been found (Forest & Saint Laurent, 1967; Scelzo, 1971, 1973; Scelzo & Boschi, 1973; Boschi *et al.*, 1992), four of them belonging to the Diogenidae.

Loxopagurus is a monospecific genus in the Diogenidae. The species *Loxopagurus loxochelis* (Moreira, 1901) is endemic to the southwestern Atlantic Ocean, being one of the most common hermit crabs that inhabit shallow littoral waters off Brazil, Uruguay and Argentina, down to a depth of 30 meters (Boschi *et al.*, 1992; Melo, 1999). The biology of this species is reasonably well known (Moreira, 1901; Forest & de Saint Laurent, 1967; Scelzo & Boschi, 1973; Scelzo, 1976; Rieger & D’Incao, 1991; Martinelli *et al.*, 2002). The life cycle of *L. loxochelis* has been studied recently in the Ubatuba region (São Paulo State, Brazil), where the occurrence of exobionts on occupied shells, shell utilization pattern, relative growth,

and spatial distribution have been investigated (Martinelli & Mantelatto, 1998, 1999; Mantelatto & Martinelli, 2001; Mantelatto *et al.*, 2004). Some aspects of the reproductive biology, particularly larval development (Bernardi, 1986), spermatophore morphology (Scelzo *et al.*, 2004), and molecular phylogeny (Mantelatto *et al.*, 2002) have been examined in recent years.

Comparisons of the functional morphology of genitalia and subsequent sperm transfer and storage mechanisms among taxa can provide useful information on phylogenetic relationships and evolutionary divergence, especially in the Decapoda (Bauer, 1986, 1991). Cladistic, molecular systematics, developmental genetics, sperm morphology, larval morphology and the fossil record are the main research fields used in the classification of recent crustaceans (Martin & Davis, 2001). The description and comparison of sperm ultrastructure are termed "spermiocladistics" (Jamieson, 1991), and according to Tudge (1997), the "use of spermatozoal ultrastructure in taxonomy and phylogeny is now firmly established as a valid means of investigating phylogenetic relationships in various animal phyla."

Mature decapod spermatozoa are aflagellate, immotile and have an uncondensed nucleus (Krol *et al.*, 1992). The noncaridean, pleocyemate decapods share a common sperm form consisting of a large acrosomal vesicle, a posterior nucleus of variable density, intervening cytoplasm containing mitochondria, microtubules, lamellar structures, and often centrioles, and a variable number (from three to many) of arms or spikes. The arms may be composed of nuclear material (higher Brachyura), microtubules (Anomura), or both (Majidae in Brachyura) (Tudge, 1992, 1997), and in most anomurans the arms have a cytoplasmic origin (Hinsch, 1991; Tudge *et al.*, 2001; Tudge & Scheltinga, 2002). Centrioles lie between the acrosome and nucleus and persist in the mature spermatozoa of some anomuran species. The conclusion from sperm ultrastructure that the Anomura *sensu stricto* are the sister group of the Brachyura (Jamieson & Tudge, 2000) is

endorsed by somatic cladistic study (Scholtz & Richter, 1995). The basic sperm morphology among the Anomura is similar to that found in the Brachyura, but the main differences lie in the acrosome shape and zonation (Tudge, 1992, 1995a, b).

This is the first ultrastructural description of the sperm of *Loxopagurus*. The Diogenidae is one of 13 anomuran families for which the sperm morphology of at least one representative has been described (Tudge, 1992, 1995a, b, 1997; Tudge & Jamieson, 1991, 1996a, b; Tudge *et al.*, 1998, 1999, 2001; Jamieson & Tudge, 2000, and references therein; Tudge & Scheltinga, 2002). The Diogenidae is an anatomically diverse family and exhibits a range of sperm morphologies intermediate between the Paguridae and Coenobitidae which may indicate a diphyletic origin for this family (Tudge, 1995a, b, 1997). At present, 21 species belonging to six genera in the Diogenidae are described for sperm ultrastructure (see Table 3 in Jamieson & Tudge, 2000, and additionally Matos *et al.*, 1993 and Manjón-Cabeza & García Raso, 2000).

This study describes and illustrates the ultrastructure of the spermatozoa of *Loxopagurus loxochelis* and compares it to that of other investigated members of the Diogenidae and Paguroidea in general.

MATERIALS AND METHODS

Hermit crabs *Loxopagurus loxochelis* were caught from 1999 through 2002 (Scelzo *et al.*, 2002) off Mar del Plata (Buenos Aires Province, Argentina) at depths between 5 and 20 meters. Adult individuals were transported alive to the laboratory at Mar del Plata National University, Argentina (UNMdP).

Small fragments of testes, *vasa deferentia* and ejaculatory ducts were fixed for 3–4 hours in 3% glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) containing 1–3 % sucrose. After three 15-min washes in phosphate buffer, the samples were postfixed for 80 minutes in 1% osmium tetroxide in phosphate buffer, washed in buffer and then processed for scanning (SEM)

and transmission (TEM) electron microscopy. Fragments of the terminal ampulla destined for SEM were dehydrated in an ascending series of acetones, critical-point dried, sputter-coated with gold, and examined in either a Jeol JSM 820 (Cádiz University, Spain) or a JSM 6460 LV (Mar del Plata National University, Argentina) scanning electron microscope. For TEM, samples of the reproductive system were dehydrated through an ascending series of alcohols (ethanol) 50–100% and embedded in Spurr's epoxy resin (Spurr, 1969). Thin sections (80 nm) stained with uranyl acetate and lead citrate (Reynolds, 1963) was examined in a Jeol 1200 EX transmission electron microscope operated at 80 kV.

RESULTS

Spermatophores were observed at the light and SEM level. The spermatophores, which are between 100 and 600 μm in size, elongate and pedunculate, have a small, flattened spherical ampulla on top of a long thin stalk. A double spermatophore ribbon is attached to the internal lining of the *vas deferens* by the base or pedestal (Fig. 1A, B). Each ampulla contains many spermatozoa (Fig. 1C). For a detailed description of the spermatophore morphology see Scelzo *et al.* (2004).

The free spermatozoa are medium-sized cells (approx. 6 μm in length) with an obvious ovoid acrosome vesicle occupying most of the cell (Fig. 1C–E). When viewed dorsally, an operculum is seen on top of the acrosome vesicle, but when viewed ventrally the sperm cells have a granular area of cytoplasm and nucleus interrupted centrally by an ovoid perforatorial chamber. Three short arms (usually only one or two are visible) extend from blunt projections of the spermatozoal outline (Fig. 1C–E). These blunt vertices are often apparent even when the accompanying arms are not visible.

At the TEM level, the three components of the spermatozoa are clearly visible (Fig. 2A–C). A large, densely staining, slightly elongate ovoid acrosome vesicle occupies the apical pole of the cell, an amorphous, electron-lucent,

nucleus occupies the opposite pole, with the heterogeneous cytoplasm intervening and displaced laterally around the acrosome vesicle (Fig. 2A, B). In longitudinal section (LS) through the mid-line of the cell, the spermatozoa average 6.7 μm in length ($n = 3$) and 4.5 μm in width ($n = 3$), including any projections of the nuclear material (excluding the arms).

The acrosome vesicle, in LS, has a mean length of 3.3 μm and mean width (at widest diameter) of 2.8 μm ($n = 7$), giving it an average length to width ratio of 1.2. This extended ovoid-shape is consistent throughout all spermatozoa recorded for this species, and they have a shallow conical operculum and a large, deep perforatorial chamber opposite this (Fig. 2B). Both structures are seen to be circular when viewed in transverse section (TS) (Fig. 2D) or at the light microscope level. The maximum diameter of the operculum averages 1.7 μm ($n = 4$) and 80 nm, or less, in thickness, and it is divided into an anterior (or upper) electron-dense region and a less dense granular region posterior to this. This latter region appears finely striated at higher magnification (Fig. 2E). The combined acrosomal and plasma membranes were often slightly disassociated from the operculum in this region and appear as a convoluted, but not broken, membrane separated from the electron-dense operculum.

The rest of the acrosome vesicle contents are arranged in four concentric zones (Fig. 2B, C): 1) a subopercular zone, 2) an inner acrosome zone, 3) an outer acrosome zone, and 4) a peripheral acrosome zone. Each of these zones is of differing appearance and electron density.

1. The subopercular zone basically fills the interior of the large, flat cone that is the operculum and has the appearance of a homogeneous finely granular matrix. Anteriorly, it is separated from the underside of the operculum by a thin electron-lucent band, and posteriorly it abuts the perforatorial chamber (Fig. 2B, C, E).

2. The inner acrosome zone extends through the centre of the acrosome vesicle thinly surrounding the perforatorial chamber. It is subjacent to the subopercular zone, at which point it is most extensive, and then attenuates into a thin

Fig. 1. Scanning electron micrographs of spermatophores and spermatozoa of *Loxopagurus loxochelis*. A, group of spermatophores (**sp**) in the *vas deferens*. B, each single spermatophore consists of terminal ampulla (**am**), stalk or peduncle (**st**), and pedestal (**pe**). C, open ampulla showing spermatozoa (**sz**). D, E, detail of sperm cells. **a**: acrosome vesicle region, **n**: nuclear region, **ra**: radial microtubular arms.

region posteriorly. It is of similar appearance to the subopercular zone but sometimes is slightly more electron-dense (Fig. 2B–D).

3. Exterior to the inner acrosome zone, and forming a thick, moderately electron-dense ring, is a zone termed the outer acrosome zone. It extends from the apical end of the acrosome vesicle (subjacent to the operculum), posteriorly, to terminate at the base of the acrosome

vesicle, encircling the perforatorial chamber opening. It has similar granularity and electron density to the subopercular and inner acrosome zones, but in some micrographs can be seen to be made up of radiating lighter and darker lines, at best, and more usually irregular dark and light patches (Fig. 2B–E).

4. The peripheral acrosome zone extends around the periphery of the acrosome vesicle,

Fig. 2. Transmission electron micrographs of *Loxopagurus loxochelis* spermatozoa. A, oblique sections through several sperm cells. B, longitudinal section of entire spermatozoa. C, oblique section through acrosome vesicle and cytoplasm. D, transverse section of the acrosome vesicle. E, anterior end of the acrosome vesicle (longitudinal section). **a**: acrosome vesicle, **c**: centriole, **cr**: cytoplasmic ring, **iaz**: inner acrosome zone, **m**: mitochondria, **mt**: microtubules, **n**: nucleus, **o**: operculum, **oaz**: outer acrosome zone, **p**: perforatorial chamber, **paz**: peripheral acrosome zone, **soz**: subopercular zone. **Arrows**, transverse and oblique sections of microtubular arms.

exterior to the inner and outer acrosome zones, from the operculum to the base of the perforatorial chamber. This zone is a homogeneously granular, yet very thin electron-pale zone which is barely distinguishable in many micrographs (Fig. 2C, D).

The perforatorial chamber is a large invagination into the posterior region of the acrosome vesicle. At its widest point, it is 1.2 μm in diameter ($n = 6$), and extends into the acrosome vesicle for approximately 2.4 μm ($n = 2$). Light and TEM observations indicate that the perforatorial chamber has a circular outline when viewed in TS (Fig. 2D), and its contents are moderately electron-dense irregular patches of coarse granularity on an electron-lucent background (Figs. 2B–D; 3D, F, G). The smooth internal wall of the perforatorial chamber is not broken by any projecting microvilli, fingers or septae. An indistinct boundary, without any visible membrane, separates the perforatorial chamber contents from the subjacent cytoplasm.

The cytoplasm forms an enveloping layer, or ring, around the base of the acrosome vesicle (Figs. 2A, B; 3E–G). The cytoplasm is thickest laterally as a 1.2 μm ($n = 4$) high ring or collar, and attenuates directly below the perforatorial chamber. It is composed of a heterogeneous assemblage of moderately electron-dense organelles and other sub-cellular structures. These organelles include many spherical, acristate or sparsely cristate, electron-lucent mitochondria (concentrated in the lateral ring), irregular membrane systems (including the occasional almost regular whorl-like arrangement), various granules and vesicles, and bundles of microtubules (Figs. 2B, C; 3E–G). These bundles are the internal regions of the three microtubular arms (Fig. 3A, C, F, G) that are visible in transverse section in the extracellular matrix around the sperm cells (Figs. 2A, B; 3C). Some thickened cytoplasmic membranes are often directed posteriorly into the nuclear material for considerable lengths. In the finely granular cytoplasm directly below the perforatorial chamber, opening is sometimes visible a single centriole (pre-

sumably one of a pair), although it is often very diffuse in appearance and difficult to discern any details of its microtubular arrangement (Fig. 3D, E).

In many of the sperm cells, a small region of anastomosing tubules and vesicles are visible between the acrosome vesicle membrane and the plasma membrane immediately anterior to the cytoplasmic ring (Fig. 3F, G). The tubular contents of these bulges are continuous with the cytoplasmic ring, but did not appear to extend into it. The small tubules in this region are larger (approx. 0.04 μm , or greater) than standard microtubules (approx. 0.02 μm), and they are generally consistent in diameter and moderately electron-dense. These tubules and vesicles were observed in both TS (Fig. 3F) and LS (Fig. 3G) of the sperm cells and usually appeared as a filled bulge or bubble emerging externally from the acrosome vesicle. In many TSs of the acrosome vesicle, this patch of tubules deforms the acrosome vesicle inwards, disrupting the normally circular profile of the acrosome (Fig. 3F, G).

The nuclear material is an amorphous, posteriorly directed plume of electron-lucent matrix, broken occasionally by small patches of more electron-dense granules and thickened cytoplasmic membranes. The nucleus is always bounded by a thick, electron-dense membrane, constituted by both the nuclear and plasma membranes (Figs. 2A, B; 3D–G).

DISCUSSION

The spermatozoal morphology exhibited by *Loxopagurus loxochelis* shows characters (symplesiomorphies) that are shared by other paguroids, and indeed, by many anomurans (Tudge & Jamieson, 1991, 1996a, b; Tudge, 1992, 1995a, b, 1996, 1997; Tudge *et al.*, 1998; Jamieson & Tudge, 2000, and references therein). These include a complex concentrically zoned acrosome vesicle capped by an electron-dense operculum and posteriorly penetrated by a perforatorial chamber, and three or more microtubular arms emanating from the cyto-

Fig. 3. Transmission electron micrographs of *Loxopagurus loxochelis* spermatozoa. A–C, longitudinal sections of radial microtubular arms (**ra**). D, E, partial views of the acrosome vesicle (basal part) (**a**) and nucleus (**n**), showing one centriole (**c**) in the subacrosomal cytoplasm below the perforatorial chamber (**p**) (the inset in E is an enlarged micrograph of the centriole). F, G, oblique sections of spermatozoa bearing tubule-containing cytoplasmic bulges (**tb**) that disrupt the normally regular profile of the acrosome. **cr**: cytoplasmic ring, **iaz**: inner acrosome zone, **m**: mitochondria, **mt**: microtubules, **o**: operculum, **oaz**: outer acrosome zone, **paz**: peripheral acrosome zone, **soz**: subopercular zone. **Arrows**, sections of microtubular arms.

plasm of the sperm cell. A spherical to oblong-ovoid or cylindrical acrosome vesicle shape predominates in the Anomura, and length:width ratios of approximately 1.0 or more have been recorded in all investigated anomuran species (Tudge, 1995a, 1997) with the exception of *Pylocheles* in the Pylochelidae (Tudge *et al.*, 2001). *Loxopagurus*, with a length:width ratio of 1.2, falls well within the bulk of the paguroid hermit crabs. In all other anomurans investigated for sperm ultrastructure, three or more microtubular arms are composed of very visible bundles of microtubules, which often can be linked directly with the same structures within the cytoplasm (Tudge & Jamieson, 1991; Tudge, 1992, 1995a, b; Tudge *et al.*, 1998; Jamieson & Tudge, 2000, and references therein). In this regard, *Loxopagurus* is no different from these other anomurans.

In overall sperm morphology, *Loxopagurus loxochelis* is most like that described for the diogenid genus *Calcinus* (Tudge, 1995a, b), and to a slightly lesser extent to the genus *Strigopagurus* (Tudge, 1996). The general sperm cell dimensions, operculum shape, subopercular zone presence and shape, and the number, appearance and arrangement of the other acrosome zones are most similar to those previously described for three species of *Calcinus*. *Loxopagurus* sperm cells lack the defining autapomorphy of *Calcinus*, which is the splitting of the anterior end of the perforatorial chamber into many obvious fingers. Also, it does not share the presence of microvillar projections in the interior wall of the perforatorial chamber as seen in all other investigated diogenid species (with the exception of a single member in the genus *Cancellus*) and members of the Coenobitidae. This absence of microvillar projections is a character shared with all investigated Paguridae, Parapaguridae and Lithodidae (Tudge, 1997; Tudge *et al.*, 1998). If the faint striations that are sometimes observed in the outer acrosome zone are indicative of a more typical acrosome ray zone, then it shares this distinctive character with all coenobitids and the genera *Dardanus* and *Diogenes* in the Diogenidae (Tudge, 1992, 1995a, b, 1997).

A less convincing similarity is seen with the sperm morphology described for *Strigopagurus boreonotus*. The sperm of *Loxopagurus* share the general opercular shape and the presence and arrangement of the subopercular, inner acrosome, outer acrosome and peripheral acrosome zones. However, both the acrosome vesicle and perforatorial chamber are more spherical in the former species, and more importantly, *Strigopagurus* sperm possess small microvillar projections in the posterior end of the perforatorial chamber.

The only unique character observed in the sperm of *Loxopagurus* is the vesicle or bulge on the side of the acrosome filled with small tubules. This vesicle is most probably an artifact or possibly a parasitic bacterial infection. The small tubules appear, at least superficially, to resemble the Rickettsia bacteria, *Wolbachia* species, previously found in the cytoplasm of reproductive cells (both sperm and eggs) of other arthropods (Johanowicz & Hoy, 1998; Van Der Geest *et al.*, 2000), including crustaceans (*Wolbachia* web page: <http://www.wolbachia.sols.uq.edu.au/hosts.cfm>), but they are here much smaller than general rickettsial bacteria. Further investigations may reveal the exact nature of these tiny tubules.

What does the ultrastructure of the spermatozoa of *Loxopagurus* reveal about the relationship of this genus to other diogenid hermit crabs? The spermatophore morphology of *Loxopagurus* is most similar to that of the diogenid genus *Clibanarius* (see Scelzo *et al.*, 2004), but the spermatozoal data do not support this relationship more than other diogenid genera like *Calcinus* or *Strigopagurus*. *Clibanarius* sperm possess two good apomorphies (possibly autapomorphies), the particular bulb shape of the perforatorial chamber and the obvious dense perforatorial ring around the posterior bulb of the perforatorial chamber (Tudge, 1992, 1995a, b, 1997). They also possess obvious and numerous microvillar projections in the perforatorial chamber, of which *Loxopagurus* sperm are lacking.

In conclusion, *Loxopagurus loxochelis* sperm morphology does not demonstrate any

clear apomorphies, but seems to separate it from the other genera in the Diogenidae by the absence of their defining sperm characters (e.g. anterior perforatorial fingers and microvillar projections). It appears most similar to that of the genus *Calcinus*, and more superficially *Strigopagurus*, although its spermatophore morphology seems to indicate a closer relationship with *Clibanarius*. The combination of spermatophore and spermatozoal characters clearly places it in the Diogenidae, but also supports its monotypic status. Recent molecular evidence (Mantelatto *et al.*, 2002) places *Loxopagurus* close to another unusual diogenid genus *Isocheles*; a view first presented by Forest (1964) when he erected the genus *Loxopagurus*. Investigation of the spermatophore and spermatozoal ultrastructure of *Isocheles* will be very revealing and should be a good test for the suggested molecular relationship.

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