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Running Head: **Sperm ultrastructure of *Pagurus excavatus***

Spermatophore and spermatozoal ultrastructure of the Mediterranean hermit crab *Pagurus excavatus* (Paguridae, Anomura, Decapoda)

TINA TIRELLI^{1*}, MARCO GAMBA¹, DANIELA PESSANI¹ AND CHRISTOPHER C. TUDGE²

¹ Life Sciences and Systems Biology Department, University of Torino - Via Accademia Albertina, 13 – 10123 Torino (Italy);

² Biology Department, American University, Washington, D.C., 20016-8007, USA and Department of Invertebrate Zoology, National Museum of Natural History, Smithsonian Institution, Washington DC, 20013-7012, USA

*Corresponding author: santina.tirelli@unito.it; ph: +39 011 6704538; fax: +39 011 6704508

Abstract

The ultrastructure of the spermatophores and spermatozoa of the Mediterranean hermit crab Pagurus excavatus are described, using transmission electron microscopy. The size of the different parts of the spermatophore and spermatozoa are given and their ultrastructure described and compared to similar data already present in the literature for other hermit crabs. The morphology and ultrastructure of the spermatophore and spermatozoa of P. excavatus are species-specific, clearly distinguishing the species from the others already described. The spermatophore and spermatozoa show some similarities with these produced by other representatives of the genus. In particular, the tripartite spermatophore is divided into two halves by the lateral ridge and the spermatozoa are composed of a cylindrical acrosomal vesicle capped by the operculum. At the base of the acrosomal vesicle, there is the thin cytoplasm, the large nucleus and three arms positioned to form a 120° angle between each other. The present description is an important additional step allowing for better understanding of the relationships among the different hermit crab taxa.

Keywords: morphology, description, reproductive products, Crustacea Decapoda

INTRODUCTION

Recently, the morphology of spermatozoa and spermatophores have been studied extensively in the Decapoda (Latreille, 1803) and provide useful information on phylogenetic relationships and evolutionary divergence (see Bauer 1986, 1991; Jamieson, 1991; Kronenberger *et al.* 2004; Jamieson & Tudge, 2000; Tudge, 2009). In particular, spermatophores and spermatozoa show a species-specific morphology, that has been successfully used in recent phylogenetic studies (Tudge 1997; Tirelli *et al.*, 2008, 2010).

The male reproductive system of Decapoda consists of a pair of testes from which a pair of vasa deferentia (divisible into regions with different functions) emanate; ending via genital openings located on the coxa of the fifth pereopods. These openings may be simple gonopores, elevated papillae, or complex copulatory structures (McLaughlin, 1983; Tudge and Lemaitre, 2004, 2006). The testes and the vasa deferentia are located dorsally or laterally to the gut and may be surrounded by the hepatopancreas (McLaughlin, 1983). In the majority of the Decapoda they are located in the cephalothorax, while Paguroidea have the reproductive in the pleon (McLaughlin, 1983). The testes produce spermatozoa, enveloped by spermatophores in which they wait to be transferred to females (Subramoniam, 1995; Hess & Bauer, 2002).

In *Pagurus* species described up to now, the male reproductive show the same patterns, described for Decapoda and Paguroidea (McLaughlin, 1980, 1983; Krol *et al.*, 1992; Fantucci and Mantelatto, 2011), but they present a great difference in the gross morphology of the vasa deferentia. These last are relatively shorter and straighter (Fantucci and Mantelatto, 2011, Scelzo *et al.*, 2010) than in described Diogenidae, in which ducts are proportionally longer and mostly helicoidal (Scelzo *et al.*, 2004; Tirelli *et al.*, 2006, 2007, 2008; Amadio and Mantelatto, 2009; Mantelatto *et al.*, 2009b). This difference in length may be a consequence of the lack in Paguridae of of the two regions responsible for lengthening of spermatophore stalk (Matthews, 1957; Greenwood, 1972). The genital openings of the *Pagurus* species (Fantucci and Mantelatto, 2011)

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are simple gonopores, differing from hermit crabs species without sexual tubes in the large number of setae surrounding the gonopore itself. These setae have been supposed to have a sensory function during mating, to provide better adhesion to the female gonopore, and to act as sensors during the transfer of spermatophores to the partner (Manjón-Cabeza and García Raso, 2000; Tudge and Lemaitre, 2004).

The spermatophores of decapods show a great morphological diversity, with different characteristics in different groups (Subramoniam, 1993). On the whole they show 3 different principal morphologies: spherical and small spermatophores, present in Brachyura; tubular in most Macrura (now Astacidea and Achelata); and pedunculate (or stalked) in Anomura (see Krol *et al.*, 1992 and Tudge, 2009 for reviews). In Anomura in general, there is a lack of information regarding ampullar ultrastructure and in particular ampullar suture line morphology. Publications correctly illustrated the lateral ridge as the point of dehiscence of the two ampulla halves in a variety of hermit crabs and other anomurans, including Paguridae (Tudge, 1999b). In particular, regarding the Paguridae, the ampulla wall and lateral ridge ultrastructure had been previously described only for *Pagurus bernhardus* (Linneus, 1758), *Pagurus hirtimanus* (White, 1847) and *Pagurus prideaux* (Leach, 1815) by Tudge (1999a, b). More recently it had been described in *Pagurus exilis* (Scelzo *et al.*, 2010) and in *Pagurus criniticornis* and *Pagurus brevidactylus* (Fantucci & Mantelatto, 2011).

Also the spermatozoa show a great structural and morphological diversity (Subramoniam, 1982; Felgenhauer & Abele, 1991; Tudge, 2009). The spermatozoa are constructed of a large acrosomal vesicle with multiple concentric layers, a posterior, amorphous nucleus, an intermediate cytoplasm and 3 to many lateral arms. In Anomura, the acrosome is oblong (Jamieson, 1991) or spherical (Tudge, 1992; Tudge & Scheltinga, 2002) in shape and has a conspicuous apical operculum. In the area between the cytoplasm and nucleus 3 or more microtubular arms have their origin (Jamieson,

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1991). The association of the sperm morphology and ultrastructure with that of other parts of the reproductive system (e.g. testes, vasa deferentia, and spermatophores) is important to better understand the reproductive biology of this taxon. Moreover, the great diversity found in recent studies of the morphology of spermatozoa and spermatophores, showing characteristics particular to members of different groups, allow us to conclude these structures are taxonomically specific (see Tudge, 1991; Subramoniam, 1993; Santos & Mantelatto, 2011). Thus, together with other taxonomic criteria, they can be used to elucidate taxonomic and phylogenetic problems (Tirelli *et al.*, 2008; Mantelatto *et al.*, 2009; Tudge, 2009; Tirelli *et al.*, 2010).

The hermit crab family Paguridae (Latreille, 1802) is comprised of 76 genera and approximately 542 species (De Grave *et al.*, 2009; McLaughlin *et al.*, 2010) and only for the genera *Anapagurus* Henderson, 1886, *Cestopagurus* Bouvier, 1897, *Nematopagurus* A. Milne-Edwards and Bouvier, 1892, *Pagurus* Fabricius, 1775, *Solitariopagurus* Tuerkay, 1986 and *Xylopagurus* A. Milne-Edwards, 1880 is the spermatophore and/or sperm morphology known (Jamieson & Tudge, 2000; Tudge, 1995a, b, 1999a; Scelzo *et al.*, 2010; Fantucci & Mantelatto, 2011).

The genus *Pagurus* is a heterogeneous pagurid group consisting of more than 170 species worldwide (Lemaitre & Cruz Castano, 2004). It is one of the most species-rich genera in Anomura (De Grave *et al.*, 2009) and is distributed in shallow waters such as littoral, subtidal and intertidal areas.

Pagurus excavatus (Herbst, 1791) is a common Mediterranean pagurid with a distribution in the Atlantic ranging from the Bay of Biscay, Spain and Portugal to Morocco, while in the Mediterranean Sea it ranges from Spain, Balearic Islands, France and Italy to Cyprus (Ingle, 1993). It mainly lives at depths between 15 and 80 m (Ingle, 1993), but specimens have been sampled down to 450 m (Chintiroglou *et al.*, 1992).

Its symbiotic relationships with sea anemones (Pax & Muller, 1962; Mainardi & Rossi, 1969; Ross, 1979; Doumenc *et al.*, 1985; Chintiroglou *et al.*, 1992), reproductive behaviour under laboratory conditions (Bastock, 1967; Hazlett, 1968), female sexual maturation and reproductive

cycle (Mura & Cau, 2002; Mura *et al.*, 2006) are well known, but no exhaustive description of spermatophore and spermatozoa is available for this species, except a concise description of the external morphology of the spermatophore under light microscopy (Tudge, 1999a).

Therefore, the aim of this paper is to expand the knowledge of the reproductive morphology of *P. excavatus* by studying the spermatophore and sperm cell ultrastructure, and comparing them with similar structures in other members of the family Paguridae.

MATERIALS AND METHODS

Specimen

One sexually mature specimen (indicated by adult shield length and gonopores present) of *Pagurus excavatus* was collected by trawling during the Cruise ARSA 0395, organized by the Instituto Espanol de Oceanographia in 1994, in the Gulf of Cadiz, off South West Spain, Mediterranean Sea. The sample was generously gifted by Prof. Antonio Medina to one of the authors (C.T.). After collection, fragments of immediately dissected testes and reproductive tract were preserved in glutaraldehyde (2.5% in sea water) for 24 hours. Then the samples were prepared for TEM observations according to standard protocols described in Tirelli *et al.* (2006, 2007).

Measurement of the different layers making up the spermatophore wall and the different regions of the spermatozoa were done from micrographs.

RESULTS

Spermatophore

The tripartite spermatophore is composed of an ampulla raised on a short stalk with a pedestal at its base. The ampulla is divided into two halves (valves), separated by a line of dehiscence, the lateral ridge. See Tudge 1995a, b and 1991a for a full description with illustrations.

The whole ampulla wall is made up of 3 layers (Figure 1A, B). The first, the inner layer (il), is finely fibrillar and electron-dense. The outer layer (ol), is made up of two strata (Figure 1C): both

being finely granular, and homogeneous but with the outer stratum being slightly more electron-dense and with an irregular exterior edge compared to the inner stratum. Between the inner and outer layers of the ampullar wall there is a homogeneous, finely granular middle layer (ml), showing some spherical electron-dense patches near the lateral ridge (see below). The inner and the outer layers of the spermatophore wall show a similar thickness, but the middle layer is between 6 and 10 times as thick as the others.

The lateral ridge is seen as a thickening of the ampullar wall where the two valves meet but they are separated by an irregular gap or discontinuity. At the TEM level (Figure 1A), in longitudinal section, the lateral ridge is conical in shape.

At the lateral ridge, the various layers thicken up to twice their depth in the ampullar wall (Figure 1A) and slightly modify their appearance. The outer layer appears to be more electron-dense, clearly distinguishing it from the middle layer and the exterior edge becomes more irregular. The middle granular homogeneous layer shows a large number of the electron-dense, circular and/or elliptic patches layer (Figure 1A). The inner fibrillar layer shows fibres with a different orientation (Figure 1A), not only parallel to the direction of cutting as seen in Figure 1D.

Table 1 shows the mean value and the standard deviation in the thickness of the different layers of the ampullar wall, including at the lateral ridge.

Spermatozoa

Spermatozoa (Figure 2A) are composed of a cylindrical acrosomal vesicle capped by a conical, highly electron-dense operculum. The subopercular zone occupies the bulk of the space beneath, and within, the operculum, and is quite well developed and homogeneously granular (Figure 2B). At the base of the acrosomal vesicle, there is the thin cytoplasm, the large nucleus and three arms positioned to form a 120° angle between each other. *Pagurus excavatus* total spermatozoal length is 5.5 µm, and total sperm width is 3.2 µm, (more detailed sperm cell dimensions are shown in Table 2).

The acrosome vesicle is posteriorly penetrated by the perforatorial chamber, which communicates with the adjacent cytoplasm directly (Figure 2A, C) and extends anteriorly up to the subopercular zone. The perforatorial chamber has an overall conical shape in longitudinal section, and exhibits a smooth margin in the basal portion and a minutely crenulated margin in the middle and distal portions (Figure 2A, C). The perforatorial chamber has fine longitudinal striations present in its entire length and often a series of obvious electron lucent tubules or vesicles (also orientated longitudinally) in the basal portion.

A slightly granular layer, more electron-dense than the subopercular zone and the perforatorial chamber, thinly surrounds the whole chamber. It is thinnest in the basal portion and doubles in thickness more anteriorly. This layer constitutes the inner acrosomal zone (Figure 2A and 2D). Immediately exterior to this is a reticulated acrosome zone (Figure 2A and 2D), showing an elongate kidney shape in longitudinal sections, but obviously a thick, open-ended cylinder in three dimensions. In both longitudinal and transverse sections this zone has the appearance of densely radiating bands of alternating dark and light electron-density (Figure 2A-D).

Exterior to the reticulated acrosome zone there is the outer acrosomal layer (Figure 2A and 2D). It is thin, homogeneously granular, of moderate electron-density and extends peripherally around the acrosome from the posterior end of the acrosomal vesicle to the posterior edge of the operculum.

Exterior to the outer acrosome zone there is the peripheral acrosome zone (Figure 2A and 2D), extending around the periphery of the acrosome vesicle, from the operculum to the base of the perforatorial chamber. This is an homogeneous, finely granular and moderately electron-dense zone. The cytoplasmic material surrounds the base of the acrosomal vesicle and a portion protrudes into the posterior opening of the perforatorial chamber (Figure 2A and 2E). It is slightly granular and homogeneous. Some cytoplasmic bodies are present including spherical, acristate or degenerating mitochondria (Figure 2D), and the longitudinally arranged bundles of microtubules, which are the bases of the microtubular arms (Figure 2A). The electron-lucent nucleus (Figure 2A,

2E and 2F) is irregular in shape and contains fibrous material and chromatin filaments; in longitudinal section, it is as wide as the upper cytoplasmic region.

DISCUSSION

Spermatophore

As previously mentioned, the spermatophores of *Pagurus excavatus* have the classic tripartite structure typical of anomurans and their general morphology was previously described by Tudge (1995a, b; 1999a). According to Tudge (1991, 1999b), the spermatophores produced by species belonging to genus *Pagurus* are morphologically very different from any other Paguroidea Latreille, 1802. The main ampulla constitutes the largest part of the spermatophore and is cylindrical in shape and attached individually, or in multiples, to a pedestal (Mouchet, 1931; Greenwood, 1972; Tudge, 1999b). In addition, an accessory ampulla is present. The accessory ampulla is a much smaller pocket (ampulla), sometimes containing one to a few sperm cells, adjacent to where the main ampulla and stalk meet and is a typical element of the mature spermatophores of pagurids, parapagurids and lithodids (Tudge 1991, 1999a; Tudge *et al.*, 1998a); all taxa in which the spermatophores do not undergo significant stalk elongation. A plausible mechanism for accessory ampulla formation was first suggested by Mouchet (1931) and later reiterated and illustrated by Tudge (1999a).

In the present study it is evident the spermatophores show a distinct lateral ridge where the ampullar halves meet, as first described by Mouchet (1930, 1931) and later by Tudge (1991, 1999b) and Tirelli *et al.* (2006, 2007, 2008, 2010) for some coenobitids, pagurids and diogenids.

The suture line is an unelevated line around the ampulla and it is more evident in the distal portion, where it becomes a crest. It is assumed, as with other anomurans, that the ampulla in *P. excavatus* opens along the lateral ridge with a zip-like mechanism, as previously described by Tudge (1991) and Tirelli *et al.* (2006, 2008, 2010).

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The lateral ridge and the spermatophore wall ultrastructure are predominantly homogeneously granular. An homogeneously granular spermatophore wall ultrastructure is considered typical of the Paguridae and has been previously reported for *Pagurus hirtimanus* (Miers, 1880), *Pagurus prideaux*, and *Solitariopagurus tuerkayi* (as *Porcellanopagurus* Filhol, 1885) (see Tudge, 1999b). A similar condition is also recorded for the diogenid *Paguristes eremita* (Linnaeus, 1767) (see Tirelli *et al.*, 2010), the parapagurid *Sympagurus* (Smith, 1883), the lomimid *Lomis hirta* (Lamarck, 1818), the galatheids *Allogalthea elegans* (Adams & White, 1948) (see Tudge, 1995a, 1997) and *Pleuroncodes planipes* (Stimpson, 1860) (see Hinsch, 1991) and the porcellanid *Aliaporcellana suluensis* (Dana, 1852) (see Tudge, 1995a; Tudge & Jamieson, 1996). The presence of the homogeneously granular spermatophore wall in all this diversity of Anomura, outside of the Paguridae, indicates that it could be the plesiomorphic condition for this trait.

The ampullar wall ultrastructure of *P. excavatus* though, does show some similarities with some Diogenidae Ortmann, 1892 including the electron-dense patches near the ridge, the irregular edge of the outer layer and the thickness of the ridge (Tudge, 1991, 1995b, 1999a; Tirelli *et al.*, 2008, 2010).

All the above-mentioned details regarding spermatophore wall and lateral ridge ultrastructure seem to be important characters to improve our knowledge of Paguroidea reproductive products, therefore should be carefully studied.

Spermatozoa

The spermatozoa of *Pagurus excavatus* show the typical morphology of anomurans: 1) an ovoid to elongate acrosomal vesicle organized into concentric zones; 2) an electron-dense operculum capping the acrosomal vesicle; 3) a perforatorial chamber partially or wholly penetrating the acrosomal vesicle; 4) cytoplasm with degenerate mitochondria; 5) a generally diffuse posterior nucleus and 6) three or more microtubular arms emanating from the cytoplasm (see Jamieson, 1991; Jamieson & Tudge, 2000 and Tudge, 2009 for reviews).

The typical acrosome vesicle shape recorded for the Anomura, varies from spherical to cylindrical, with a length:width ratio of approximately 1 or more, as reported for all anomurans studied to date, except for the symmetrical hermit crab *Pylocheles* A. Milne Edwards, 1880 (see Tudge *et al.*, 2001). The acrosome of *P. excavatus*, is cylindrical, quite large, constitutes almost the entire sperm volume and has a length:width ratio of approximately 1.75, being in agreement with data reported in the literature (Tudge, 1995b, 1997, 1999a, b).

In particular, the sperm cells of *P. excavatus* are characterized by not having microvillar projections in the perforatorial chamber, an absence similarly reported for other Paguridae (especially belonging to genus *Pagurus*), the Parapaguridae and Lithodidae (Jamieson & Tudge, 2000; Tudge, 1999a, b, 1995a, b; Tudge & Justine 1994), and the diogenids *Loxopagurus loxochelis* (Scelzo *et al.*, 2006), *Cancellus* H. Milne-Edwards, 1836 (see Tudge, 1995a, b) and *Paguristes eremita* (Tirelli *et al.*, 2010). Furthermore, in *P. excavatus* the perforatorial chamber shows conspicuous longitudinal tubules and vesicles, especially basally. Similar tubules have already been reported for the diogenid hermit crab *P. eremita* (Tirelli *et al.*, 2010) but are more commonly observed in the Brachyura (e.g. Jamieson, 1991; Tudge *et al.*, 1994, 1998b; Jamieson & Tudge, 2000).

Pagurus excavatus, as with other members of the genus *Pagurus* already described, is characterized by the presence of one or more reticulated acrosome zones (also possibly present in the parapagurid, *Sympagurus*) (Tudge, 1995a, b, 1997; Jamieson & Tudge, 2000) between the inner and the outer acrosome zones. Furthermore the perforatorial chamber shows a conical shape in longitudinal section and penetrates to the subopercular zone. It shares this deep penetration with other members of the genus *Pagurus*, *Lithodes*, and the diogenids *Calcinus* and *Clibanarius*, in contrast to much shorter perforatorial chambers in *Diogenes*, *Dardanus* and *Coenobita* (Tudge, 1992, 1995a, b, 1997).

Another important characteristic present in the spermatozoa of *Pagurus excavatus* is the presence of the peripheral acrosome zone. This external-most acrosome zone has been previously

described in many other hermit crabs, including pagurids and parapagurids (Tudge, 1995a, b), diogenids (Tudge, 1996; Scelzo *et al.*, 2006; Tirelli *et al.*, 2010), in other anomurans including the hippids *Emerita talpoida* (Say, 1817) and *Hippa pacifica* (Dana, 1852) (see Tudge, 1995a; Tudge *et al.*, 1999), and even among some brachyurans like *Cancer pagurus* Linnaeus, 1758 (see Tudge *et al.*, 1994), *Segonzacia mesatlantica* (Williams, 1988) and *Austinograea alayseae* (Guinot, 1990) (see Tudge *et al.*, 1998b), for example.

The minimal cytoplasmic region enveloping the base of the acrosomal vesicle, with only a small portion connected with the perforatorial chamber; more or less degenerate spherical mitochondria, the characteristic “triad” pattern of the base of the three microtubular arms; and, finally, an electron-lucent and fibrillar nucleus are all fairly ubiquitous throughout the Anomura.

The overall dimensions of the spermatozoa, of *P. excavatus* (5.5 µm long by 3.0 µm wide) are much smaller than many hermit crabs described to date (e.g. *Birgus*, *Coenobita* and *Diogenes*) but larger than others (e.g. *Calcinus* and *Clibanarius*) (Tudge, 1992, 1997).

In conclusion, as reported above, both spermatophores and spermatozoa produced by *P. excavatus* show the general characteristics already described for other Paguroidea and in particular show some similarities with spermatophores and spermatozoa produced by other representatives of the genus. This may be due to the fact that, being Paguridae a very diverse and speciose taxon comprising 76 genera (De Grave *et al.*, 2009) with approximately 176 species in the genus *Pagurus* alone, our knowledge of spermatophore and sperm morphology of this genus is at the very beginning. This means that available knowledge on the group may just represent a small part of the great variety of morphologies and ultrastructures potentially developed in the taxon. Therefore, it is of primary importance to enlarge the study of the ultrastructure of pagurid spermatophores and spermatozoa, in order to increase our knowledge of their reproductive biology, and to perform a phylogeny of this family, as already done for Diogenidae (Tirelli *et al.*, 2010). This is an essential step to enlarge our understanding of such a complex taxon as the Paguridae, and its relationship to other Paguroidea and Anomura.

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Table 1. Thickness of ampulla wall and lateral ridge of *Pagurus excavatus*.

statistics	lr [μm]			aw [μm]		
	out	mid	inn	out	mid	inn
N	5	5	5	5	5	5
mean	0.69	2.53	0.38	0.17	1.18	0.25
SD	0.11	0.76	0.10	0.05	0.07	0.01

aw, ampulla wall; out, outer layer; mid, middle layer; inn, inner layer; lr, lateral ridge; N, number of lateral ridges or ampullae measured; SD standard deviation.

Table 2. Measurements of the various portions of the sperm cell of *Pagurus excavatus*.

statistics	tl [μm]	tw [μm]	per [μm]		op [μm]		nu [μm]	
			l	w	l	w	l	w
N	4	4	4	4	4	4	4	4
Mean	5.47	3.02	3.81	0,92	0.91	1.41	0.66	1.02
SD	0.91	0.04	0.69	0,09	0.25	0.32	0.04	0.23

l, length; N, number of spermatozoa measured; nu, nucleus; op, operculum; pc, perforatorial chamber; SD, standard deviation; tl, total length; tw, total width; w, width.



