

# Reproductive apparatus of the male giant hermit crab *Petrochirus diogenes* (Anomura, Diogenidae): morphology and phylogenetic implications

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**ABSTRACT:** We describe the male reproductive apparatus of the giant hermit crab *Petrochirus diogenes*, with morphological and biometric analyses of the spermatophore, the gonopore and the ultrastructure of the spermatozoa. Specimens were collected from the southern coast of São Paulo, Brazil. Morphological analyses were done using stereoscopic, light, transmission and scanning electron microscopy. The reproductive system of this hermit crab is composed of elongate and lobular testes followed by vasa deferentia that connect to the exterior via gonopores. The gonopores are ovoid and surrounded by setae, and each gonopore is composed of a membranous operculum that forms a depression constituting the gonopore opening. The gonopore constitutes a unique structure among the Diogenidae due to its number of setae. The spermatophores are tripartite, composed of a sperm-containing ampulla, a peduncle and a proximal foot. The spermatozoon has 3 main regions (acrosomal vesicle, nucleus and cytoplasm). The structure of the spermatophore indicates that this species can be considered an exception within Diogenidae with regard to spermatophore morphology and can therefore be used for phylogenetic inferences.

**KEY WORDS:** Decapoda · Reproduction · Spermatophore · Spermiotaxonomy · Gonopore

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## INTRODUCTION

Comparisons of reproductive morphology are a taxonomic tool, providing key characters for species delimitation, solving taxonomic problems and establishing phylogenetic relationships between groups of crustaceans (Mantelatto et al. 2009a, Fantucci & Mantelatto 2011). Recently, studies based on electron microscopy have intensified interest in morphological analyses of the reproductive structures of decapod crustaceans, especially in males (Manjón-Cabeza & García-Raso 2000, Amadio & Mantelatto 2009). Recent studies of the structure of spermatozoa and spermatophores among hermit crab families provide information on phylogenetic relationships (Mantelatto et al. 2009a).

The diogenid *Petrochirus diogenes* Linnaeus, 1758, one of the largest aquatic hermit crabs, is endemic to the western Atlantic and is distributed along the east coast of the USA, from North Carolina into the Gulf of Mexico, the Antilles, Venezuela, Suriname, Uruguay and Brazil (from Amapá in the north to Rio Grande do Sul in the south) (Coelho & Ramos 1972, Williams 1984, Melo 1999).

Although our knowledge of the life cycle of *Petrochirus diogenes* is incomplete, some of the biological features of this species have been described in recent years, particularly in studies carried out in the southwestern Atlantic. Previous reports have described the larval development (Provenzano 1963, 1968), feeding and chewing structures (Caine 1975), behavior related to shell occupation (Kellogg 1977, Bertini

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1997, Bertini & Fransozo 2000a), relative growth (Bertini & Fransozo 1999a), population dynamics (Bertini & Fransozo 2000b), spatial and seasonal distributions (Bertini & Fransozo 1999b), reproductive period (Bertini & Fransozo 2002) and population biology (Turra et al. 2002) of this species. Data on the morphology of the reproductive apparatus are still scant. To our knowledge, the only previous study on this topic is a description of spermatozoal ultrastructure by Brown (1966).

The male reproductive system in Decapoda is typically composed of a pair of testes connected to a pair of vasa deferentia (VD) that open to the exterior via gonopores located on the ventral side of the coxae of the fifth pair of pereopods (Matthews 1953, McLaughlin 1983, Krol et al. 1992). The VD can be externally divided into proximal, median and distal regions (McLaughlin 1983, Amadio & Mantelatto 2009). The testes and VD are generally located in the cephalothorax, but in some anomuran families, including hermit crabs, the gonads are situated in the pleon (McLaughlin 1983, Krol et al. 1992). The male reproductive apparatus of hermit crabs has a species-specific morphology and has recently been used in phylogenetic studies (Tudge 1997, Tirelli et al. 2006, 2008). Spermatozoa are bundled together with secretory materials in the VD and eventually form the spermatophores (Subramoniam 1991, 1993, Krol et al. 1992), a common means of spermatozoa transfer that has evolved among invertebrates (Tudge 1991, Subramoniam 1993). The production of these structures is habitat-related, and in marine species, the spermatophores appear to reduce spermatozoal loss (Subramoniam 1993). In addition, spermatophores provide an energy-rich substrate, allowing for spermatozoal storage over long periods (Subramoniam 1991, Scelzo et al. 2004, Mantelatto et al. 2009a).

Spermatophores play an important role in Crustacea because most crustacean spermatozoa, including those of all decapods, are aflagellate and non-motile (Felgenhauer & Abele 1991, Jamieson 1991), increasing the importance of a vehicle of transfer in the absence of an intromittent copulatory organ (Subramoniam 1993). Among Anomura, spermatophores are generally pedunculate (Subramoniam 1993, Tudge 1999b) and can be divided into 3 major regions: a sperm-filled ampulla, a stalk of variable length and a sticky foot or pedestal that affixes the spermatophore onto the female (Tudge 1991, 1999a, Subramoniam 1993). Spermatophores in anomurans display species-specific morphology in the shape of the ampulla and the length of the peduncle (Subramoniam 1984). In Diogenidae, spermatophores gen-

erally display long, thin stalks and small, spherical ampullae (Tudge 1991).

Spermatozoa morphology also varies among crustaceans, and it is difficult to identify features that characterize the entire class (Jamieson 1991, Tudge 2009). However, ultrastructural analyses have been useful in elucidating phylogenetic relationships, principally in Decapoda (Tudge 1992, 1997, 2009, Jamieson 1994). In decapods, the spermatozoon is composed of a large acrosomal vesicle with multiple concentric zones, a basal nucleus and an intermediate cytoplasm with organelles and a variable number of arms or spikes (Tudge 1992, 2009). In Anomura, the acrosomal vesicle is divided into concentric layers, delimited by an electron-dense operculum and penetrated by a perforatorial chamber, and there are 3 microtubular arms emerging from the cytoplasm or nucleus (Jamieson 1991, Tirelli et al. 2008). The spermatozoa of Diogenidae, though, are not a clear phylogenetic marker at the family level, as they share characteristics with the spermatozoa of some members of the families Paguridae and Coenobitidae (Tudge 1992).

The present study describes the male reproductive apparatus of the giant hermit crab *Petrochirus diogenes* and morphological and biometric analyses of the spermatophore, gonopore and spermatozoal ultrastructure, to provide information on phylogeny.

## MATERIALS AND METHODS

Specimens of *Petrochirus diogenes* were collected using a shrimp fishing boat equipped with double-rig trawling nets in the Enseada de Ubatuba region (23° 25' 06" to 23° 27' 42" S and 45° 05' 00" to 45° 02' 48" W), Ubatuba, São Paulo, southeastern Brazil. The live hermit crabs were maintained in the laboratory until the analysis was conducted. We selected 15 males covering the widest possible range of sizes. All procedures followed the protocols described by Scelzo et al. (2004, 2010), Amadio & Mantelatto (2009) and Mantelatto et al. (2009a).

Each individual was anesthetized by chilling, its shield length (SL) was measured (from the tip of the rostrum to the V-shaped groove at the posterior edge) with a caliper rule (0.1 mm), and its wet weight was measured using an electronic balance (0.01 g). Also, the largest diameter of both gonopores of each animal was measured using a stereomicroscope with a camera lucida. Gonopore samples for SEM analysis were isolated from the crab by removing a section (coxae) of the fifth legs, containing the gonopores.

Each individual was dissected in the dorsal position, and an incision was made in the cuticle of the pleon, exposing the hepatopancreas and the reproductive system. Spermatophores from each region of the VD were analyzed using light microscopy, and photos were obtained; 10 spermatophores from the distal portion of the VD of each animal were used for biometric analysis, and the following measures were taken using a light microscope: ampulla length (AL), peduncle or stalk length (PL), total length of the spermatophore (TL), ampulla width (AW) and peduncle width (PW) (Fig. 1). Testes, VD and spermatophores were preserved in 80% ethanol.

Data (including dry weight, shield length, gonopore diameters and spermatophore measures) were analyzed using Sigma Stat for Windows, v. 2.03, with  $\alpha = 0.05$  for all tests. The data were tested for normality, and a Pearson's correlation was performed to examine the relationship between shield length and spermatophore biometrics, which were normally distributed. Spearman's correlations were used to analyze the relationships between shield length and each of dry weight and gonopore diameter, which were non-normally distributed.

Details of gonopore and spermatophore morphology were obtained using scanning electron microscopy (SEM), and spermatozoa were analyzed using transmission electron microscopy (TEM). For SEM analysis, samples were fixed in 3% glutaraldehyde

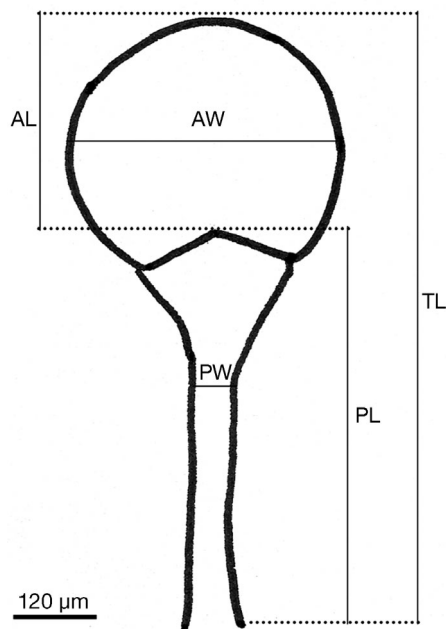


Fig. 1. *Petrochirus diogenes*. Drawing of a spermatophore showing the measurements taken: (TL) spermatophore total length, (PL) peduncle length, (PW) peduncle width, (AW) ampulla width, (AL) ampulla length

(5 d for gonopore samples; 2 h for spermatophores), transferred to phosphate saline buffer and post-fixed for 2 h in 1 to 2%  $\text{OsO}_4$  in buffer solution, dehydrated in ethanol (30%, 50%, 70%, 80%, 90% and 3 steps in 100% for 30 min each) and critical-point dried using liquid  $\text{CO}_2$ . Samples were then placed on SEM stubs, sputter-coated with gold, then observed and photographed in a scanning electron microscope (JEOL JSM5410; JEOL JS5200). TEM samples were prepared using procedures similar to those applied to the SEM samples. Following dehydration, the material was embedded in Epon-Araldite resin, cut into thin sections on an ultramicrotome Leica Ultracut S with a diamond blade and transferred to copper grids. Samples were then stained with lead citrate and uranyl acetate and observed and photographed in a transmission electron microscope (JEOL JEM100CX2).

Voucher specimens were deposited in the Crustacean Collection of the Biology Department (CCDB) of the Faculty of Philosophy, Sciences and Letters of Ribeirão Preto (FFCLRP) at the University of São Paulo, Brazil (catalogue numbers CCDB 3110 to 3116 and CCDB 3118).

## RESULTS

A total of 15 adult specimens were analyzed, and the specimens presented SL values ranging from 12.46 to 31.62 mm and averaging  $25.31 \pm 5.54$  mm and wet weights ranging from 22.35 to 414.29 g and averaging  $247.35 \pm 38.98$  g (Table 1). These measurements were positively and significantly correlated with the size (SL) of the animals ( $r_s = 0.933$ ,  $p < 0.001$ ).

### Reproductive system

The reproductive system is composed of paired testes followed by a pair of VD (Fig. 2) that communicate to the exterior via gonopores located on the bases of the coxae of the fifth pair of pereopods. The testes and VD are located dorsally in the pleon, in a central position, and they are adjacent to the hepatopancreas. The testes are white, lobular and elongated on the antero-posterior axis. The VD are also white and consist of coiled tubular ducts that can be divided into 3 different regions (proximal, median and distal) according to their external morphology. The proximal region is near the testes and is coiled, the median region is also coiled but less so than the

Table 1. *Petrochirus diogenes*. Body dimensions and spermatophore measurements (n = 15). SL: shield length; W: wet weight; AL: ampulla length; PL: peduncle length; TL: total length; AW: ampulla width; PW: peduncle width

	Mean ± SD	Minimum	Maximum	Correlation to SL	
				r	p
SL (mm)	25.31 ± 5.54	12.46	31.62	–	–
W (g)	247.35 ± 138.98	22.35	414.29	–	–
AL (µm)	263.53 ± 65.75	120	420	0.745	0.001
PL (µm)	524.9 ± 154.9	170	820	0.709	0.003
TL (µm)	728.3 ± 201.3	270	1180	0.736	0.002
AW (µm)	263.4 ± 65.4	110	400	0.806	<0.001
PW (µm)	48.5 ± 16.07	20	110	0.483	0.068

proximal region, and the distal region is straight and ends in a gonopore near the anterior part of the pleon (Fig. 2).

### Gonopores

The gonopores are oval, completely surrounded and covered by plumose and simple setae (Fig. 3A,B), and have a calcified gonopore cover (Fig. 3C). Each gonopore presents a probably calcified operculum (gonopore cover), totally covered by plumose setae (Ps) (according to Watling 1989) that originate in the anterior region and end in a depression, which constitutes the gonopore opening (Fig. 3B,C). In the posterior region of the gonopore, there is a group of simple setae (Ss) (according to Watling 1989) bordering the opening (Fig. 3B–E).

The 2 gonopores occupy a significant portion of the coxae of the fifth pair of pereopods. The largest diameter of the gonopores ranged from 1.39 to 5.04 mm, averaging  $3.61 \pm 0.79$  mm (n = 15) for crabs measuring 12.46 to 31.62 mm SL. The correlation between the largest diameter of the gonopores and SL was positive ( $r_s = 0.908$ ,  $p < 0.001$ ).

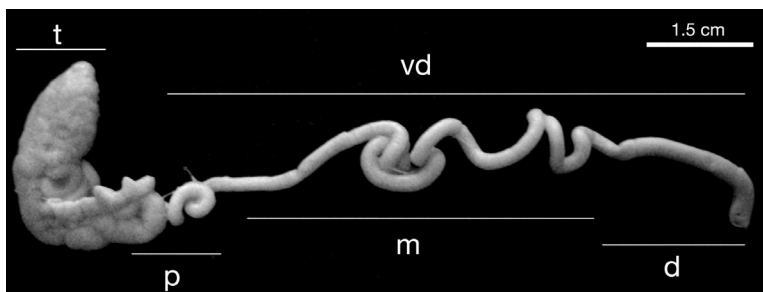


Fig. 2. *Petrochirus diogenes*. Reproductive system of the adult male, with (t) testis and the (p) proximal, (m) median and (d) distal regions of the (vd) vas deferens

### Spermatophores

The spermatophore is pedunculate and is divided into 3 major regions: a distal spherical sperm-filled ampulla, a thin stalk and a proximal foot (Fig. 4A,B). The presence of mucus surrounding the spermatophores interfered with the SEM observations of the foot and the end of the stalk (Fig. 4C). The ampulla is larger than the stalk, which has a small projection where it joins the ampulla (Fig. 4A). The am-

pulla is nearly spherical (Fig. 4A,B), with a suboval shape laterally, and consists of 2 valves joined along their lateral edges by a low lateral ridge (Fig. 4D). The external surface of the ampulla is irregular, with small depressions likely formed either by the tight packaging of spermatozoa within the ampulla or by the process of dehydration for SEM, or both (Fig. 4D). The lateral ridge is externally formed by a thickening in the wall of the ampulla where the 2 valves meet, through which the valves likely release spermatozoa (Fig. 4D).

We observed no significant morphological differences between the spermatophores produced in different regions along the VD. Spermatophores from the proximal region had slightly flatter spherical ampullae and shorter, thicker stalks. Spermatophores from the median region had stalks that were intermediate in length and thickness and spherical ampullae indistinguishable from those on spermatophores from the distal region of the VD.

### Spermatozoon

The spermatozoon has 3 main regions: an ovoid acrosomal vesicle, a basal nucleus and an amorphous intermediate cytoplasm (Fig. 5A,B) with 3 arm-shaped extensions (Fig. 5C). The acrosomal vesicle is divided into an inner acrosome zone, an acrosome ray zone and an outer acrosome zone (Figs. 5A,B,D). The inner acrosome zone constitutes the central column of the spermatozoon and is the less electron-dense layer of the acrosomal vesicle (Fig. 5A,B,D). The outer acrosome zone surrounds the acrosomal vesicle except for the operculum (Fig. 5A,B). The acrosomal vesicle has an electron-dense operculum at its apex, followed by a granular subopercular zone, which extends posteriorly into the inner acrosome zone

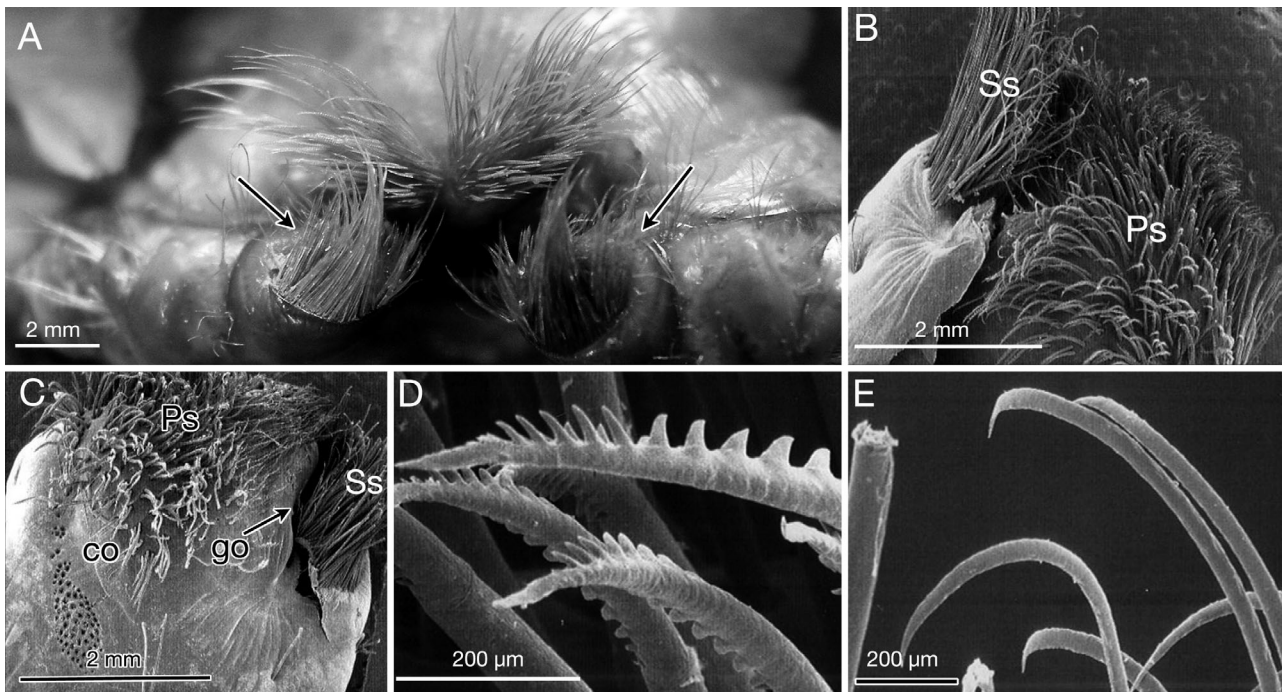


Fig. 3. *Petrochirus diogenes*. Gonopore morphology. (A) Detail of the fifth pair of pereopods; arrows indicate the gonopores. Scanning electronic micrographs: (B) Gonopore completely covered by (Ps) plumose and (Ss) simple setae. (C) Gonopore with (co) the calcified gonopore cover with (Ps) plumose setae, (go) the depression that constitutes the gonopore opening and the group of (Ss) simple setae bordering this opening. (D) Detail of the plumose setae. (E) Detail of the simple setae

(Fig. 5A,B). The vesicle is also penetrated at its base into its center by a cylindrical perforatorial chamber, which extends along the apical-basal axis (Fig. 5A–C) and presents microvillar projections on its wall (Fig. 5B,D). The nucleus is located at the base of the spermatozoon and consists of an amorphous structure with a granular aspect, and its border to the cytoplasm is not well defined, due to the absence of a membrane (Fig. 5A,B). The cytoplasm, located in the base of the sperm cell, is continuous with the perforatorial chamber (Fig. 5A,B,D) and contains organelles such as mitochondria (Fig. 5D).

## DISCUSSION

### Reproductive system

The male reproductive system is located dorsally in the pleon, as in other Anomura (McLaughlin 1983, Krol et al. 1992), whereas in other decapod crustaceans, the reproductive organs are located in the cephalothorax (McLaughlin 1983, Krol et al. 1992, Amadio & Mantelatto 2009). The basic composition of the reproductive system, including a pair of testes connected to a pair of VD that open to the exterior via gonopores, is similar to that seen in other decapods

(Matthews 1953, McLaughlin 1983, Krol et al. 1992, Amadio & Mantelatto 2009), as is the apparent division of the VD into 3 different regions (McLaughlin 1983, Amadio & Mantelatto 2009). The testes are elongated and lobular as in other hermit crabs, such as *Diogenes pugilator* Roux, 1828 (Manjón-Cabeza & García-Raso 2000), *Calcinus tubularis* L., 1767 (Tirelli et al. 2006), and *Calcinus tibicen* Herbst, 1791 (Amadio & Mantelatto 2009).

Morphological and histological features of the VD are associated with spermatophore development in that spermatophore formation is determined by the secretory, muscular and glandular activities of the VD (Mouchet 1931). The shape of the spermatophore is also related to and influenced by the shape of the VD (Mouchet 1931). A detailed understanding of the morphology and histology of the VD is therefore important, and histological analyses of this structure are needed to obtain a better comprehension of the reproductive system.

### Gonopores

The male gonopores of *Petrochirus diogenes* are ovoid in shape, the same pattern described in other species of Diogenidae, such as *Calcinus tibicen*

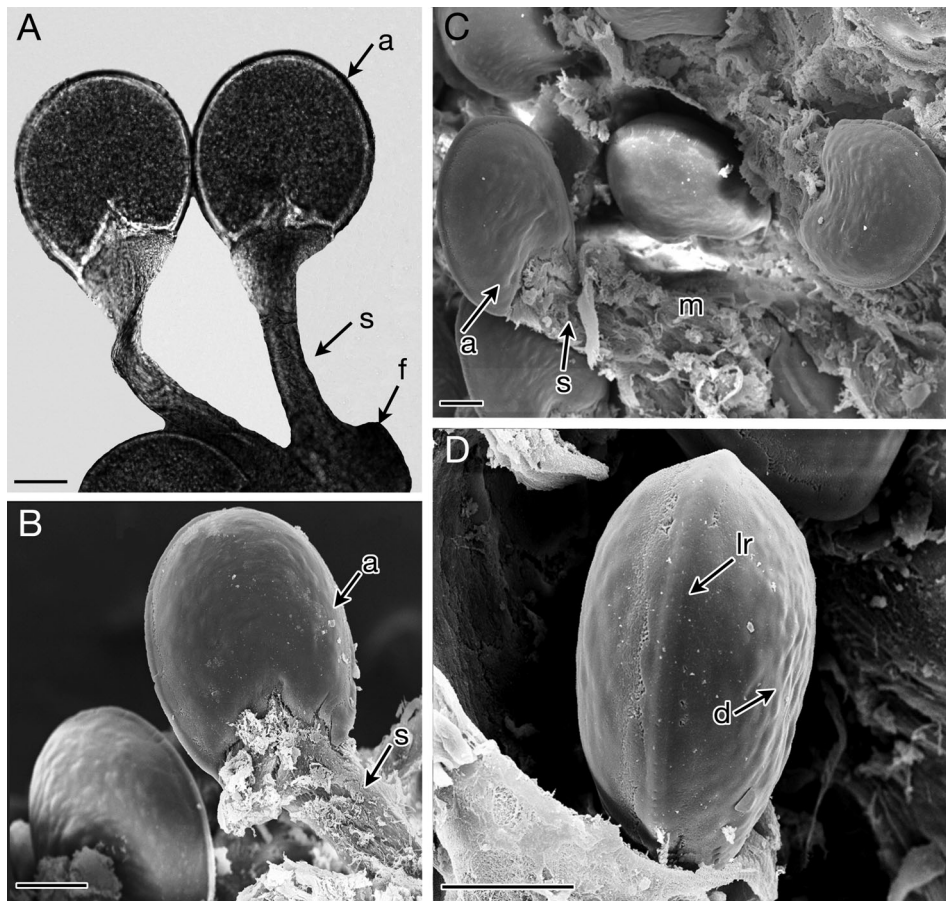


Fig. 4. *Petrochirus diogenes*. Spermatophore morphology. (A) General morphology, including (a) the ampulla, (s) the stalk and (f) the proximal foot. Scanning electronic micrographs: (B) General morphology of the spermatophore, including (a) the ampulla and (s) the stalk. (C) Mass of spermatophores with (a) ampulla, (s) stalk and (m) mucous surrounding them. (D) Detail of the ampulla, showing the (lr) lateral ridge and (d) depressions in the wall. Scale bars = 100  $\mu$ m

(Amadio & Mantelatto 2009), *Isocheles sawayai* Forest & Saint Laurent, 1968 (Mantelatto et al. 2009b), and *Loxopagurus loxochelis* Moreira, 1901 (Scelzo et al. 2004). However, the gonopores of *P. diogenes* differ significantly from those of other species with respect to the number of associated setae. Whereas the gonopores of *P. diogenes* are completely surrounded by setae, the gonopores of some species, such as *Calcinus tibicen* (Amadio & Mantelatto 2009) and *Clibanarius scolopetarius* Herbst, 1796 (Santos & Mantelatto 2011), exhibit few or no setae. Other species, such as *I. sawayai* (Mantelatto et al. 2009b), *L. loxochelis* (Scelzo et al. 2004), *Clibanarius erythropus* Latreille, 1818 (Tirelli et al. 2007), and *Diogenes pugilator* (Manjón-Cabeza & García-Raso 2000), exhibit an intermediate number of gonopore setae, but these are still far fewer than in *P. diogenes*.

During reproduction, the setae surrounding the gonopore might play an important role as sensors of the female cephalothorax and genital openings in addition

to facilitating the transfer of spermatophores to females during copulation (Manjón-Cabeza & García-Raso 2000, Tirelli et al. 2007), thereby increasing transfer efficiency and reproductive success. The presence of transfer structures (other than setae) in Diogenidae is known only in the genera *Paguristes* Dana, 1851, and *Pseudopaguristes* McLaughlin, 2002, in which the first and second pairs of abdominal pleopods in the male are modified as gonopods that act as transfer agents (Tudge & Lemaitre 2004). Some other families, including Coenobitidae and Paguridae, have specialized spermatozoa transfer structures called sexual tubes, which consist of prolongations of either the VD or the male gonopores (Tudge & Lemaitre 2004), but these are also not found in *Petrochirus diogenes*.

There is limited information in the literature about how the spermatophores are transferred to the female. We only know that in the superfamily Paguroidea, spermatophores are placed externally on the exoskeleton or in the surface of the gastropod shell

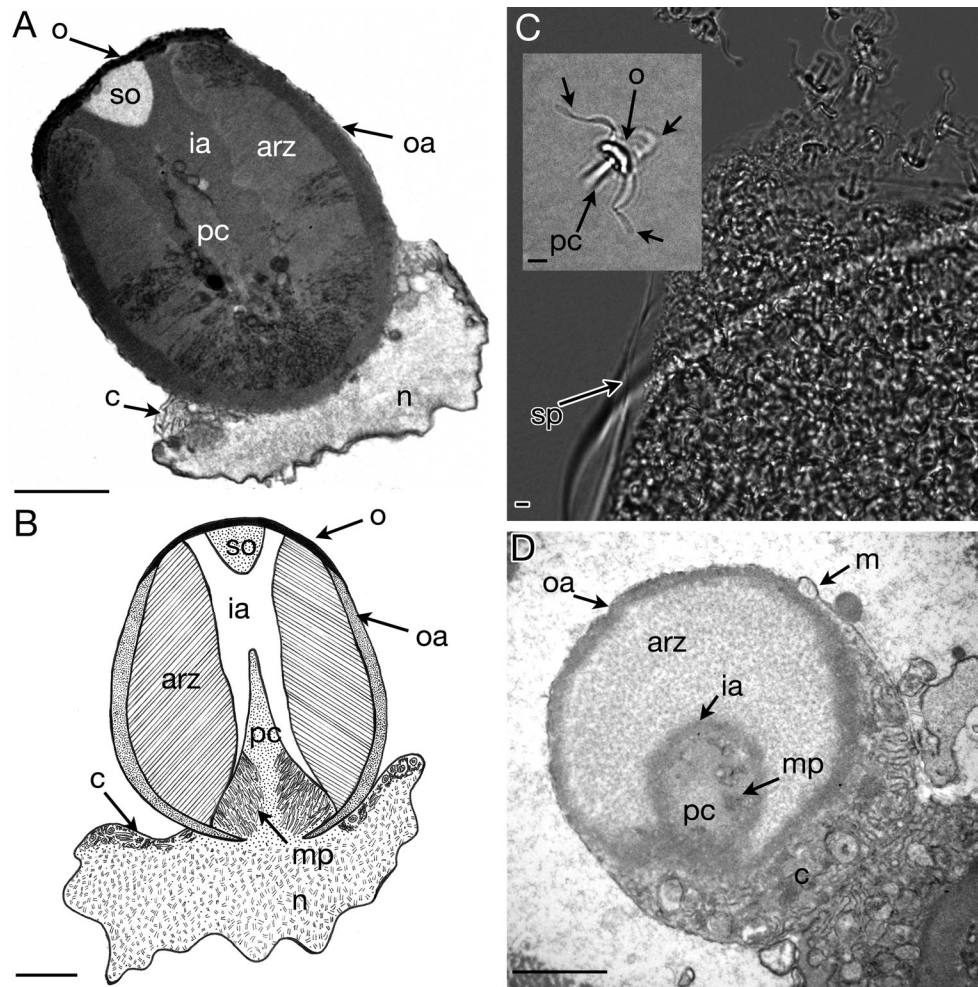


Fig. 5. *Petrochirus diogenes*. Spermatozoon morphology. (A) Tangential section of the spermatozoon, showing the ovoid acrosomal vesicle, (n) the nucleus and (c) the cytoplasm (scanning electron microscopy). (B) Spermatozoon illustration showing (mp) microvillar projections of the perforatorial chamber. (C) Spermatophore releasing the mass of spermatozoa; on the detail, arrows indicate the 3 arms (light microscopy). (D) Transversal section of the spermatozoon, showing (m) mitochondria in the cytoplasm. ia: inner acrosome zone; oa: outer acrosome zone; arz: acrosome ray zone; o: operculum; so: subopercular zone; pc: perforatorial chamber. Scale bars = 2  $\mu$ m

inhabited by the female (Matthews 1953, Tudge 1991, Hess & Bauer 2002).

The positive correlation between the SL measurements and gonopore diameter indicates that the size of the gonopores is directly proportional to the size of the hermit crab, as reported for other diogenid species (Amadio & Mantelatto 2009, Mantelatto et al. 2009b, Santos & Mantelatto 2011). This pattern means that an intimate relationship during ontogeny occurs between hermit crab size and reproductive body dimensions.

### Spermatophores

Spermatophore morphology has now been described for 25 species in 11 genera of diogenid hermit

crabs, which represent 6% and 55% of all known species and genera, respectively. The present study provides the first detailed description of spermatophore morphology and morphometry for hermit crabs of the genus *Petrochirus*.

The spermatophores of *Petrochirus diogenes* are morphologically similar to those previously described in several other diogenid species, including *Calcinus minutus* Buitendijk, 1937, *Calcinus latens* Randall, 1839, *Clibanarius corallinus* H. Milne Edwards, 1848, and *Clibanarius virescens* Krauss, 1843. The spermatophores of these species consist of a small and spherical ampulla and a long, thin stalk (Tudge 1991).

The external morphology of the lateral ridge in *Petrochirus diogenes* is also similar to that observed in other species of Diogenidae, including *Calcinus*

*tibicen* (Amadio & Mantelatto 2009), *C. tubularis* (Tirelli et al. 2006), *Isocheles sawayai* (Mantelatto et al. 2009a) and *Diogenes pugilator* (Tirelli et al. 2008). In these species, the lateral ridge consists of a thickening of the wall of the ampulla at the border along which the 2 valves are joined.

Despite these similarities, the analyses using light and electron microscopy in the current study revealed that the spermatophore of *Petrochirus diogenes* presents an exception among Diogenidae. Although it has a spherical ampulla and a thin stalk, the spermatophore in this species presents a relatively large ampulla in relation to the length of the stalk. Similar proportions are seen in the spermatophores of *Calcinus tibicen* (Amadio & Mantelatto 2009) and *C. minutus* (Tudge 1991). This similarity suggests that this particular spermatophore morphology is a shared trait in these species, a possibility that is corroborated by a recent molecular phylogeny (Mantelatto et al. 2009b).

Other Diogenidae, such as *Diogenes gardineri* Alcock, 1905, and *Isocheles sawayai*, also show spermatophores with large ampullae relative to the stalk (Tudge 1991, Mantelatto et al. 2009a). However, in these species, this proportion is seen in width measurements, not length, as the stalk of the spermatophore is very long in these species, as discussed below.

Although continuous characters are generally not used for phylogenetic analyses of morphological data, new methods have recently incorporated algorithms for their analysis (Goloboff et al. 2006). However, some continuous characters, such as spermatophore measurements, should not be used to infer relationships or, as proposed by Scelzo et al. (2004), as a criterion to characterize and distinguish species, because the dimensions are strongly associated with the individual size and cannot be used alone in phylogenetic comparisons (Amadio & Mantelatto 2009, Santos & Mantelatto 2011). Nonetheless, proportions between spermatophore regions can indicate shape (such as an ovoid or cylindrical ampulla or an ampulla much bigger than the stalk) and can be used to infer phylogenetic relationships.

The measurements of the spermatophore of *Petrochirus diogenes* confirm that the ampulla is relatively large in relation to stalk length. The mean AW is 5.4-fold greater than PW in this species, and the mean PL is less than twice AL (Table 2). Similar proportions between the ampulla and the stalk are also found in other species of Diogenidae, such as *Calcinus tibicen* (Table 2) (Amadio & Mantelatto 2009). Some diogenid species show different spermatophore proportions, however. In *Isocheles sawayai*, the relationship between AW and PW is similar to that found in *P. diogenes*, but PL is much larger than AL (Mantelatto et al. 2009a). In *Loxopagurus loxochelis*, also a member of Diogenidae, the difference between AW and PW is not as large, and PL is more than twice AL (Scelzo et al. 2004).

Analysis of the male reproductive apparatus in combination with molecular, behavioral and population studies enhances our understanding of the life history of this species and provides information on phylogenetic relationships. In this vein, considering the phylogenetic tree in Fig. 6, based on molecular data, it is apparent that *Calcinus tibicen* is phylogenetically closer to *Petrochirus diogenes* than to *Isocheles sawayai* or *Loxopagurus loxochelis*. This proximity can be corroborated by spermatophore morphology, as *C. tibicen* exhibits spermatophore morphology that is more similar to that of *P. diogenes* than to the spermatophore morphologies of the other 2 species, as described above.

In contrast, diogenid genera such as *Dardanus* Paulson, 1875, and *Clibanarius* Dana, 1852, showed proximity to *Petrochirus diogenes* in the phylogenetic tree, but their spermatophore morphology presents different features compared to those of *Calcinus* Dana, 1851. The genus *Dardanus* has few species with described spermatophores, but *Dardanus megistos* Herbst, 1804, has a spermatophore with a thick, short stalk and a small, ovoid ampulla (Tudge 1991), which differs from the spermatophores of *P. diogenes*. The genus *Clibanarius*, besides its proximity to *P. diogenes*, has species with 2 types of spermatophores. Species such as *Clibanarius sclopetarius*

Table 2. Comparisons of the spermatophore measurements of some representatives of the family Diogenidae. AW: ampulla width; PW: peduncle width; PL: peduncle length; AL: ampulla length; n: sample size

Species	AW/PW	PL ( $\mu\text{m}$ )	AL ( $\mu\text{m}$ )	n	Source
<i>Petrochirus diogenes</i>	5.43	524.9 $\pm$ 154.9	263.53 $\pm$ 65.75	15	Present study (Table 1)
<i>Calcinus tibicen</i>	3.37	130 $\pm$ 45	89.8 $\pm$ 17.8	30	Amadio & Mantelatto (2009)
<i>Isocheles sawayai</i>	3.63	498 $\pm$ 280	102 $\pm$ 33	70	Mantelatto et al. (2009a)
<i>Loxopagurus loxochelis</i>	2.22	278 $\pm$ 102	116 $\pm$ 23	298	Scelzo et al. (2004)



and *C. vittatus* Bosc, 1802, show non-tripartite spermatophores, without stalks or feet, but the ampulla morphology follows that described for Diogenidae (Hess & Bauer 2002, Santos & Mantelatto 2011). Other species, such as *C. erythropus* and *C. corallinus*, show tripartite spermatophores with small, spherical ampullae and thin, long stalks (Tudge 1991), so the non-tripartite pattern can be considered as a secondary loss in this genus (Santos & Mantelatto 2011). Species from the family Coenobitidae, represented by *Coenobita compressus* H. Milne Edwards, 1837, in the phylogenetic tree, also have different spermatophores, with large spherical ampullae and short thick stalks, showing a robust appearance (Tudge 1991).

Overall, despite these proportional morphometric differences among the spermatophores in the family Diogenidae, these structures are generally pedunculated, with the size of the ampulla varying in relation to the size of the stalk. This morphology allows a finer

taxonomic resolution of the species, in addition to differentiating the spermatophores of these species from those of species in other families, such as Paguridae (Fig. 6).

The positive correlations between SL and weight, and between SL and spermatophore measurements (AL, PL, TL, AW and PW) indicate that the size of the spermatophores is directly proportional to the size and weight of the animal. This pattern has also been found in other species in Diogenidae, including *Calcinus tibicen* (Amadio & Mantelatto 2009) and *Clibanarius sclopetarius* (Santos & Mantelatto 2011).

### Spermatozoon

The characteristics of the spermatozoon of *Petrochirus diogenes* found in the present study are similar to those described by Brown (1966), especially with respect to the tri-radial shape of this structure, its immobility and the presence of a multilayered acrosomal vesicle with an apical operculum. Brown (1966) found an acrosomal vesicle comprising 3 layers: an outer dense layer with an inner tubular part surrounded by an amorphous one, a middle microtubular layer that he called the bulk of the acrosomal vesicle and a dense inner layer that extends from the apex into the proximal end. In sequence, these layers probably correspond to what we call here the acrosome ray zone surrounded by the outer acrosome zone, the inner acrosome zone as the 'bulk of the acrosomal vesicle' and the perforatorial chamber as the dense inner layer. Nevertheless, Brown (1966) did not mention the presence of a subopercular zone and found no mitochondrion.

The spermatozoon of *Petrochirus diogenes* is generally similar to those observed in other species of Decapoda in possessing an acrosomal vesicle, a basal nucleus and an intermediate cytoplasm with organelles and arms (Tudge 1992, 2009). The acrosomal vesicle with multiple layers delimited by an electron-dense operculum, the perforatorial chamber penetrating this acrosomal vesicle and the 3 microtubular arms are all characteristics found in spermatozoa among anomurans (Jamieson 1991, Tirelli et al. 2008).

These 3 characteristics mentioned above are also found in the spermatozoa of other Diogenidae, such as *Strigopagurus boreonotus* Forest, 1995 (Tudge 1996), *Diogenes pugilator* (Tirelli et al. 2008), *Calcinus tibicen* (Amadio & Mantelatto 2009) and *Clibanarius sclopetarius* (Santos & Mantelatto 2011). The acrosomal vesicles of diogenid species range from ovoid, in *Dardanus crassimanus* H. Milne Edwards,

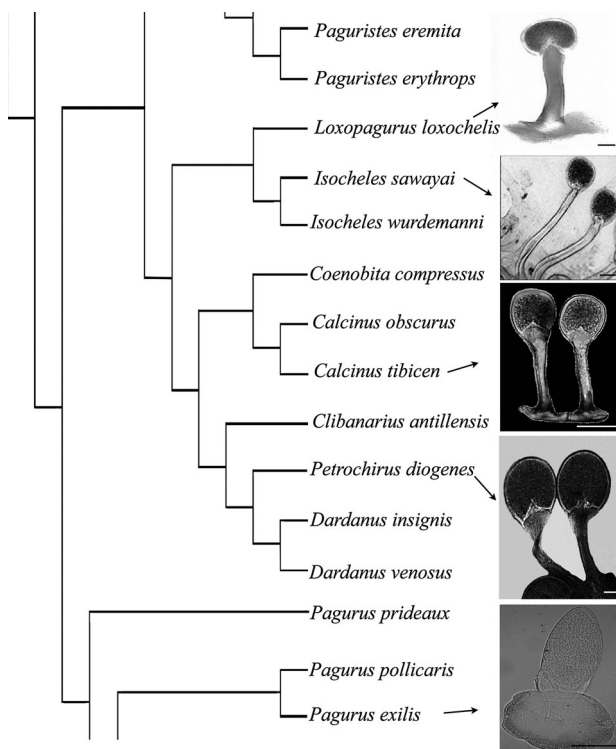


Fig. 6. Part of a phylogenetic tree based on molecular characters (modified from Mantelatto et al. 2009b) with the spermatophore morphology of representatives of the family Diogenidae (*Loxopagurus loxochelis* [modified from Scelzo et al. 2004], *Isocheles sawayai* [modified from Mantelatto et al. 2009a], *Calcinus tibicen* [modified from Amadio & Mantelatto 2009] and *Petrochirus diogenes* [present study]) and Paguridae (*Pagurus exilis* [modified from Scelzo et al. 2010]). Scale bars = 70  $\mu$ m

1836 (Tudge 1992), to spherical, in *C. tibicen* (Amadio & Mantelatto 2009), or almost spherical, such as those of *Clibanarius corallinus* (Tudge 1992). All diogenid species with described spermatozoa show a conical operculum at the anterior pole of the spermatozoon, sometimes followed by a subopercular zone, as in *Diogenes custos* Fabricius, 1798 (Tudge 1992). The perforatorial chamber penetrating the acrosomal vesicle can be found in all diogenids, and its extension varies among species. It extends anteriorly from for one-third of the length of the acrosomal vesicle, as in *D. crassimanus* (Tudge 1992), up to almost the entire length of the vesicle, as in *C. tibicen* (Amadio & Mantelatto 2009).

Except for the species of the genus *Clibanarius*, all diogenid species present spermatozoa with a 3-layered acrosomal vesicle, showing an inner and outer acrosome zone and an acrosome ray zone (Tudge 1992, 1995, Tirelli et al. 2008, Amadio & Mantelatto 2009). Also, the genus *Clibanarius* shows a spermatozoon with a perforatorial chamber, divided into a posterior perforatorial bulb and an anterior perforatorial projection (Jamieson 1991, Tudge 1992, Tirelli et al. 2007, Santos & Mantelatto 2011).

This range of features suggests that there are no family-specific characteristics in the spermatozoa of this family. Moreover, some species in this family also show spermatozoa with characteristics found in the spermatozoa in the families Paguridae and Coenobitidae, including some representatives of the genera *Clibanarius* and *Diogenes* Dhillon, 1968, respectively (Tudge 1992, 1995).

Ultrastructural analyses of sperm morphology in Diogenidae are limited in terms of species diversity because most of these analyses have focused on the description of spermatozoa in only a few genera. Additional studies should therefore be done, including the analysis of the male reproductive system of other species of hermit crabs. This would improve our understanding of reproduction in these species and provide useful information on phylogenetic relationships.

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