Article

**Morphometric, Histochemical, and Ultrastructural Analysis of the Reproductive System and Spermatogenic Stages of Male Blue Crab (Callinectes sapidus Rathbun, 1896)**

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**Abstract:** The blue crab, *Callinectes sapidus* Rathbun, 1896, is considered to be a luxury meal, especially in touristic cities. It contains more than 20 types of amino acids and provides all the needed amounts for human growth. This study describes the morphology and complex differentiation in the gonadosomatic index, morphological and ultrastructure features of the reproductive system, spermatogenesis, and spermatophores structure; this is due to the need to maintain natural and fishing stocks. Mature adult male crabs (carapace length $59 \pm 7.12$; width $126 \pm 18.8$ mm) were obtained from Abu-Qir Bay from November 2018 to October 2019 and transported alive in seawater to the laboratory. The reproductive system was dissected and weighed to the nearest 0.001g using the electronic balance, and the gonadosomatic index was subsequently calculated. The morphological analysis showed the developing testes with highly compacted seminiferous tubules. Using Periodic acid–Schiff stain, the spermatophore appeared with a zigzag-shaped wall that indicates its carbohydrate constituents. Each Spermatophore consisted of an inner spermatozoal mass embedded in a matrix, whose main components were secretions 1 and 2, and an outer thin acellular layer composed of secretions 3 and 4 from the anterior vas deferens (AVD). Secretions 5 and 6 (S5 and S6) also appeared with carbohydrate constituents using Mallory triple stain. The transverse section of the middle vas deferens (MVD) showed spermatophores with rod-shaped secretion S6 and granular secretions 7 (S7), forming a complex matrix between spermatophores. The secretion found in the MVD was granular, strongly acidophilic, and secreted by its highly columnar epithelium (S7). The ultrastructure showed that the testes were surrounded by a monolayer of myoid cells with an elongated nucleus, which also contained the following stages: spermatogonia, primary spermatocyte, secondary spermatocytes, and spermatids as well as spermatozoa. On the other hand, scanning electron microscope studies for fully formed spermatophore taken from the middle part of the vas deferens indicated that it is ellipsoidal in its outline with terminal stalk. Furthermore, the spermatophore was surrounded by a thick capsule of non-cellular substances and contained mature spermatozoa.

**Keywords:** *Callinectes sapidus*; male; histology; testes; vas deferens; gonad somatic index

1. Introduction

In recent years, crabs have been among the most common fresh produce in the food sector as an animal protein source with premium costs [1–3]. These are known to be good
sources of omega-3-polyunsaturated fatty acids, amino acid content, and due to their calcium content, they are recommended for pregnant women [4,5]. The major crab species are Callinectes sapidus, Charybdis feriata, Charybdis lucifera, Portunus pelagicus, Portunus sanguinolentus, Charybdis truncata, Scylla serrata, and Scylla tranquebarica [1]. The Blue crab, Callinectes sapidus, is widely reported in various Mediterranean regions [6,7]. Morphometrics and length-weight relations are often used to evaluate population characteristics [8–10] and determine the size of sexual maturity or possible variability among species in the stock assessment of commercially significant species [11,12]. The production of crabs largely depends on the quality of the broodstock, and the male reproductive system information is crucial when collecting the matured males for breeding [13].

Spermatogenesis in crabs starts with spermatogenesis multiplication and the development of primary and secondary spermatocytes. It is divided into spermatids and eventually into sperm cells [14]. Mature sperms are then transported through the vas deferens and encapsulated into spermatophores [15,16]. Generally, there are few ultrastructural reports in some of the decapods [17,18]. Although the role of several components of the spermatoozon has been investigated, there are uncertainties in others, including the numerous acrosomal surfaces. For example, the decondensed chromatin is reported to have the requisite malleability for the acrosome reaction [17]. Early findings revealed that the sperms are in a non-compact organization, with chromatin suspended in the nucleoplasm at varying degrees of decondensation, whose length is not significantly diminished [19]. Other studies indicate that the volume of the final sperm nucleus is reduced and has very compact chromatin, mainly due to an increased amount of sperm nuclear basic proteins (SNBPs), particularly histones, protamines, and protamine-like proteins [20]. Therefore, the form, structure, internal organization, the case of the nucleus, and the degree of “chromatin condensation,” mature sperm nuclei and acrosomes in “decapod crustaceans” are very distinct [20].

Although the decapod sperm maturation process is complex and different in each taxonomic community, certain key points of sperm structure are accepted by previous studies. For example, the vas deferens of Pinnotheres pisum and Pinnotheres nobilis conform to that of other brachyurans in being lined with glandular epithelium. The secretions are thought to contribute to both spermatophore formation and seminal plasma content [21]. In Maja brachydactyla [22], three different types of secretions were found, two of which are involved in the formation of spermatophores. The medial vas deferens (MVD), where spermatophores are stored, and seminal plasma is secreted, is strongly widened in Nepinnotheres pinnotheres and Pinnotheres pisum compared to those of other brachyurans [23]. Brachyurans generally secrete large quantities of seminal plasma [24]. The secretion occurs in the continuous tube of the medial and distal vas deferens (DVD) [25]. The DVD originates from the final portion of the MVD and is located ventrally to the cephalothorax, and its inside lining has simple cubic epithelium [26]. In N. pinnotheres and P. pisum, additional secretion takes place in the special appendices of the DVD. In the portunid, Callinectes sapidus [27], spider crabs M. brachydactyla [22], and Chionoecetes opilio [28], the DVD also possesses appendices (or diverticula) that produce and store seminal plasma. These “secretory accessory glands” occur along most of the length of the DVD [22]. Within the anterior vas deferens (AVD), a protein-rich substance is secreted that individually surrounds each spermatoozon (their spermatophore ground substance ‘SS-1’ that forms larger aggregates. Within the distal part of the AVD, a second secretion (‘SS-2’) fills the space between the spermatophores, which is considered to be the final step in spermatophore formation in M. brachydactyla [22]. The Golgi complexes specifically are involved in transforming the “acrosome vesicle and spermatid nucleus into mature sperm” [29]. The final acrosome shape ranges from an ill-defined acrosome in Portunidae decapods [19]. The sac-like structure near association with an anterior spike in other dendrobranchiate decapods is often distinct in other species [30].

However, in the blue crab (Callinectes sapidus), the male reproductive system, including the spermatogenic stages, is not clearly understood. This lack of clear information has been a contributing factor limiting the culture of this species in aquaculture. Moreover,
a clear understanding of the reproductive system of different fishery resources is key to the sustainable management of fisheries. Therefore, this study aimed to investigate the gonadosomatic index of male maturation seasons, morphological and ultrastructure of the spermatogenesis, sperm, and spermatophores structures. The gonadal maturation could be a significant taxonomical tool in *Callinectes sapidus*.

2. Materials and Methods

2.1. Study Area

*Callinectes sapidus* were obtained from Alexandria beach, Egypt, at Abu-Qir bay and 52 male crabs were collected each season (Autumn, Winter, Spring, Summer) from November 2018 till—November 2019, transported alive in seawater using a plastic container to the laboratory in Zoology Department, Faculty of Science, Alexandria University. Abu-Qir bay is a semi-circular basin located about 35 km northeast of Alexandria city between Lon. 30°4’ and 30°21’ east and Lat. 31°16’ and 31°28’ north, (Figure 1).

2.2. Studied Animal

In this study, adult male crabs were used once they became sexually mature [31]. The heterochelic claws finished with the first pair of pereopods, and two to four pereopods were moving legs, while the fifth pereopods were swimming legs. The abdomens were inverted T-shaped, and the third to fifth abdominal segments were fused and formed the gonopods. The collected crab samples were in inter molt stage (exoskeleton was completely calcified) [32].

2.3. Morphometric Measurements

Each male crab and its reproductive system were weighed according to [33]. Mean ± standard deviations were associated with the Student *t*-test for each male (*α* = 0.05) [34]. The dissected gonads were measured to the nearest 0.001 g by using the electronic balance, and, subsequently, the following formula calculated gonadal somatic index:

\[
\text{GSI %} = \frac{\text{Wet weight of the gonadosomatic index}}{\text{Wet weight of the gonad}} \times 100
\]

The reproductive system was differentiated macroscopically according to the color and sizes of both testes and vas deferens. Accordingly, the male gonads were assigned to four groups or stages, which are the developed, premature, early mature, and mature stages.

In each isolated specimen, a cut was done at the center of the transverse bridge to get two halves of the male system. One was used for histological examination after fixation with 5% neutral formalin. At the same time, the other side was for transmission electron microscopical studies.

2.4. Histochemical Studies

Approximately 210 crabs (*C. sapidus*) characteristics in Abu-Qir, Mediterranean Sea, were studied for total body weight (g), Length, Width (cm) and the gonado-somatic index % in each male from (November 2018–November 2019) as shown in (Figure 2). Small fragments taken from isolated parts of testis and vas deferens from different stages of maturity were processed for histochemical studies; the isolated parts were fixed in a 5% neutral formalin solution. The standard method of dehydration, clearing, and paraffin embedding was followed [35]. Sections of 5–6 µ were done. The sections were stained with Mallory’s triple stain, Masson’s trichrome, Heidenhain’s iron-hematoxylin, and PAS.
Figure 1. Map of the Mediterranean Sea showing the distribution of the blue crabs (Callinectes sapidus) by red dots [7].
Figure 2. Total body weight (g), Length, Width (cm), and Gonado-somatic index % in males of *C. sapidus* characteristics in Abu-Qir (June–November 2019), means ± SE Columns superscripted by different letters within the same axis are significantly different (*p* < 0.05).

Also, the stereoscopical examination was made for the isolated mature system using Olympus stereoscope SZ7 in the electron microscope unit in the Faculty of Science, Alexandria University.

2.5. Electron Microscopic Study
2.5.1. Scanning Electron Microscopy Study

For electron microscopy scanning, the chosen parts of male reproductive organs were fixed in 2% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.2) for 2 h at 4 °C. After rinsing in cacodylate buffer, the tissues were posted, fixed in 1% osmium tetroxide in the same buffer for 1 h at 4 °C, and rapidly washed in cacodylate buffer. The material was then transferred to an aqueous solution of 1% thiosemicarbazide for 15 min, followed by an aqueous 1% osmium tetroxide for 30 min at 4 °C. After rinsing in distilled water, the organ was dehydrated in graded ethanol series by subsequent exchanges of the following dilutions in distilled water as follows:

- 25% E.T.O.H., 1 × 5 min for delicate specimens;
- 50% E.T.O.H., 1 × 5 min; 75% E.T.O.H., 1 × 5 min (specimen can be stored overnight at 4°C at this step);
- 95% E.T.O.H., 1 × 5 min;
- 100% anhydrous E.T.O.H.* 3 × 10 min (less time may be required for monolayers).

Caution: ETOH and acetone are hygroscopic, and freshly opened solvent or stock stored in a desiccator should be used for final 100% exchanges to avoid “wet” ETOH, which can induce drying artifacts [36].
2.5.2. Transmission Electron Microscopic Study

Filtered seawater was used from the collection point for osmotic maintenance. Each vas deferens was split into three parts (proximal, middle, and distal) with small parts of each portion, and sections of the testes were fixed for 3 h in 2% paraformaldehyde/2.5% glutaraldehyde fixative (pH 7.2) at 4 °C. It was then washed in 0.1 M phosphate buffer, postfixed for 1 h in 0.1 M phosphate buffer and 1% osmium tetroxide buffer (at room temperature), then washed five times in buffer. The tissues were dehydrated (30, 50, 70, 80, 90%, and 2 changes of absolute alcohol) via graded ethanol series. After dehydration, penetration was carried out using a series of mixtures of propylene oxide and embedded in Epon resin. Ultrathin sections (50 nm) were cut using Leica UC7 ultramicrotome (Leica Microsystems, Wetzlar, Germany), and contrasted with 2% aqueous uranyl acetate (20 min) and 2% lead citrate in NaOH 0.1 N (7 min). The material was photographed under a TEM operating (Philips CM100; Philips Electron Optics, Eindhoven, the Netherlands) at 80 kV [37].

The prepared parts were examined in an Electron Microscope, (JEOL-JSM-5300, Akishima, Japan) in the unit of the “Faculty of Science Alexandria University.”

3. Results

3.1. Morphometric Characters

The average carapace width was 126 ± 18.80 mm, while the highest value was 179 ± 9.71 mm and the lowest was 82 ± 5.17 mm. The carapace length showed an average of 59 ± 7.12 mm, while the maximum carapace length was 79 ± 3.04 mm. Moreover, the weight of 100 males *C. sapidus* showed an average value at 145.9 ± 5.98 g., while the highest value was 329.3 ± 11.52 g. The lowest value was 48.9 ± 2.93 g. The morphological examination of the reproductive system taken through the different seasons of the year appears in accordance with the morphometrical study of the size, length, and width of the cephalothorax. The values of GSI varied from 2.65 to 5.56% for males of total body weight ranging from 147.02 to 207.6 g, carapace length with two peaks at 14.24–34.83 cm with significant differences during the seasons in Abu-Qir 2019 (Figure 2). The best values of total body weight (g), Length, Width (cm), and GSI % in males of *C. sapidus* in the Spring season without significant differences in Autumn, and the lowest values were in Summer with a significant difference.

3.2. Spermatophore

Electron scanning microscopic study for fully formed spermatophore taken from the middle part of the vas deferens indicated that it is ellipsoidal in its outline with a prominent terminal stalk (Figure 3a). A glance at the results (Figure 3b) showed its distal stalk. A magnified photo for the outer wall of the spermatophore showed its surface topography wrinkled on one side and smooth surface on the other side (Figure 3c). The spermatophore’s wrinkled outer surface had deep grooves that appeared as regular elevated parts (Figure 3d). SEM examinations for opened stalked spermatophores showed many preserved sperms aggregated together with the accompanying testicular secretions, which appeared to wrap them (Figure 3e). The mature sperm were multi-stellate with short radial arms. The number of these arms cannot easily be counted because of the umbrella-like structure formed from the testicular secretions (S1 and S2) (Figure 3f).
Figure 3. Electron micrograph scanning showing: fully formed spermatophore (Sph) (a). Electron scanning micrograph showing: the terminal stalk of the spermatophore (arrow) (b). Electron scanning micrograph showing: spermatophore with the smooth outer surface (white arrow) and wrinkled surface (black arrow) on both sides (c). An enlarged part from the previous figure showing: the wrinkled outer surface of the spermatophore with deep grooves (black arrow) (d). Electron scanning micrograph showing the spermatophore with its internal spermatozoa (black arrows) (e). An enlarged part from the previous figure showing spermatozoa (SZ) with radial arms (black arrow) (f).
3.3. Spermatogenesis

The ultra-thin sections showed that the testes were surrounded by an outer layer of muscles consisting of a monolayer of myoid cells (Figure 4). A clear desmosomal junction holding myoid cells together were illustrated (Figure 4a). Next to the myoid cell, spermatogonium is a large cell in the germinal zone near the seminiferous tubule’s basal lamina. Most spermatogonial cells that were round to oval-shaped possess a thin cytoplasm and a large oval nucleus containing small heterochromatin blocks that aggregate at the nuclear envelope (Figure 4b). Next to the spermatogonia, the primary spermatocytes undergo 6 stages through prophase for transfer into second spermatocytes. They are spherical cells with a centrally located spherical nucleus; their size and nuclei would not change during meiotic divisions. Preleptotene spermatocytes have granular homogeneous cytoplasm and few organelles, as well as nuclei with small chromosomes. Small mitochondria with few and poorly developed cristae are found throughout the cytoplasm, containing slightly electron-dense material. The small vesicular endoplasmic reticulum comprises isolated, irregular cisternae containing low-electron-density material (Figure 4c). Individualized chromosomes were condensed into strands in leptotene spermatocytes, and the cytoplasm had a concentric membrane system. Two centrioles migrating to the opposite poles of the cell were noticed. The centriole is made of microtubules wrapped in an extension of the plasma membrane with a ring of 9 microtubules doublets surround a central pair of microtubules (Figure 4d).

The zygotene spermatocytes were of the same size as the leptotene. They are discernible from leptotene spermatocytes by the existence of denser and “long cord-like chromosome” blocks in the oval nucleus. A slightly prominent and electron-dense nucleolus-like body or nuage was noticed at the cytoplasm (Figure 4e). The Pachytene stage showed paired chromosomes with synaptonemal complexes inside the nucleus and many mitochondria in its cytoplasm (Figure 4f).

Chromosomes were depicted as a cluster of thick chromatids in the nucleus center during the diakinesis stage, which was similar to the zygotene (Figure 5). The scant cytoplasm contained few organelles, such as rough endoplasmic reticulum and free ribosomes. In this stage, dispersion of nuclear envelope and desmosome between the cells is still present in (Figure 5a). The nucleus of the secondary spermatocytes was filled with nucleoplasm and tightly condensed chromosomes located at one side of the cell. The cytoplasm was less electron-dense and contained several irregular endoplasmic reticulum cisternae with light electron-dense material and a large number of small-rounded mitochondria (Figure 5b). Early spermatids were spherical and slightly polarized, with the nucleus at one pole and the cytoplasm at the other (acrosomal pole), where the proacrosomal vesicle was constructed. The nucleus was spherical and contained granular chromatin, which appeared as condensed clumps in the nucleoplasm and linked to the nuclear envelope. Nuclear pores facing the acrosomal pole were visible on the nuclear envelope (Figure 5c). The endoplasmic reticulum differentiated, and the proacrosomal vesicle developed at the acrosomal pole in spherical to oval cells-like mid-spermatids. The decondensation of chromatin was the first change seen in mid-spermatids. As a result, chromatin in the nucleus was homogeneous, with only a few small, condensed clumps (Figure 5d). Late spermatids were highly polarized cells with a voluminous proacrosomal vesicle and a reduced half-moon-shaped nucleus at the nuclear pole. The nuclear envelope was fused with the plasma membrane, forming a thick, electron-dense membrane (Figure 5e). The spermatophore was stored inside the middle vas deferens. It was surrounded by a thick capsule with one layer of non-cellular substances and contained mature spermatozoa. The spermatozoa possessed the acrosomal region as a spherical organelle embedded in the nuclear cup (Figure 5f).
Figure 4. Transmission electron micrograph of Callinectes sp. testis showing (a). basal part of seminiferous tubule with myoid cell, nucleus (N), nuclear pores (dashed arrows), the cellular junction (arrows), contracted fibers (star), endoplasmic reticulum (ER), and basement membrane (BM). Spermatogonia rest on the basement membrane (BM) with nucleus (N) contains dispersed euchromatin, many dense mitochondria (M), and rough endoplasmic reticulum (rER) (b). Preleptotene stage with nucleus (N), small chromosome (arrow), double nuclear envelope (dashed arrow), few mitochondria (M), and small vesicular endoplasmic reticulum (ER) (c). Leptotene stage with nucleus (N), clear thread-chromosomes (arrow), centriole (dashed-arrow), vesicular rough endoplasmic reticulum (rER), and annulated lamellae (dashed arrow) (d). Zygotene stage with nucleus (N) has intertwined thick chromosomes, paired cords (black arrows), poor mitochondria (M), vesicular rough endoplasmic reticulum (star), nuage (white arrow), secondary lysosome (Ly2) (e). The Pachytene stage has a nucleus (N), chromosomes have synaptonemal complexes (arrows), poor small mitochondrion (M), free ribosomes (R), and myelin body (star) (f).
Figure 5. Diakinesis stage has a nucleus (N) with thick chromatids, dispersed nuclear envelope (arrows), scant cytoplasm contains few rough endoplasmic reticulum (rER) and free ribosomes (R) (a). Secondary spermatocytes stage with small nucleus (N), nucleoplasm (star), tightly condensed chromatin (arrow), vesicular endoplasmic reticulum (rER), small rounded mitochondria (M), and ribosomes (R) (b). Early spermatid with large euchromatic nucleus (N) and heterochromatin blocks in poor cytoplasm (c). Mid spermatid has a nuclear pole (NP) with small clumps of chromatin, acrosome pole (AP) with acrosomal vesicle, ribosomes in poor cytoplasm (R), lytic mitochondria (M), vesicular endoplasmic reticulum (rER) (d). Late spermatid stage with half-moon heterochromatic nucleus (arrow) and lytic mitochondria (M) in poor cytoplasm (e). Spermatophore surrounded by a thick capsule (C), mature sperm (Sz), acrosome (star) nucleus (N), and cell boundaries (arrows). Mature spermatozoa have a fully developed cylindrical acrosomal core between the nuclear cup and acrosomal region and a thin layer of lamellae. The radial arms are continuous with the nuclear cup. The apical cap of sperm is a specialized, thickened structure (f). The acrosomal region’s body surrounds an “acrosomal tubule, which extends from the apical cap proximally to the central region bordering the nuclear cup.
The apical cap was present as a very electron-dense band (Figure 6), which resembled a disk shape, and it was the only part of the acrosomal region not enclosed by the nuclear cup (Figure 6a). The sections of the acrosomal vesicle showed dark and paled distinct concentric layers surrounding the acrosomal tubule. The innermost layer was described as the acrosomal ray’s layer. Exteriory, the membrane could be traced over the surface of the acrosomal vesicle (Figure 6b).

**Figure 6.** Spermatozoa with apical cap (AC), acrosomal membrane (dashed arrow), acrosome (A), acrosomal tubule (AT), lamellae (white arrow), operculum (Elbow arrow), and cell boundaries (black-arrow) (a). Spermatophore has spermatozoa with acrosomal tubule (AT), acrosomal membrane (black arrow), acrosome (A), and cell boundaries (white arrows) (b).

3.4. **Vas Deferens**

The vas deferens began as a voluminous compact coiled mass behind the stomach. The anterior vas deferens (AVD) with middle and posterior vas deferens (PVD) were convoluted tubular (Figure 7).

**Figure 7.** A photomicrograph of a transverse section of the testes of Callinectes sapidus showing evacuated duct with sperm and Secretion 1 (S1) (red color) and secretion 2 (S2) with (green color) (Masson trichrome stain) (A). A photomicrograph of a transverse section of the distal part of anterior vas deference Notice; spermatophore (Sph) with S5 (Star) and S6 secretions (black arrow) (Mallory triple Stain) (B).
4. Discussion

Currently, studies that have investigated the reproductive mechanism of the Blue crab are restricted to a few reports [27,38]. Besides, these studies are generally old, which merits further investigations to confirm the findings and gain new insights.

In decapods,
Transverse sections of the testes stained with triple stains showed two different testicular secretions. The first one (SI) appeared fluid and was secreted by the formed epithelial lining of the seminiferous ductulus. It appeared highly acidophilic and was intermingled with the somewhat compacted spermatozoa, while the other secretion (S2) was basophilic and appeared to be secreted by the nurse cells that are interfered with the spermatozoa (Figure 7a). At the distal part of AVD, the fully formed spermatophores were embedded in a secretion (5 and 6) (Figure 7b).

Stereoscopical examination of the vas deferens (Figure 8) showed that the AVD is finally tubular, highly convoluted, and filled with pinkish secretions. The MVD was more enlarged, slightly convoluted, filled with gelatinous secretion, and was highly transparent. The PVD appeared somewhat slight with less coiling and white secretion (Figure 8a). The spermatophores of the adult sexually matured male crab are stored in the MVD, and they appear spherical to oval-shaped, as shown in (Figure 8b). Each spermatophore consisted of inner spermatozoa mass included in a matrix, whose main components were secretion 1 and 2 (S1 and S2), and an outer thin acellular layer composed of secretion 3 and 4 (S3 and S4) secreted from the AVD. Using PAS, the spermatophore appeared with a zigzag-shaped wall indicating carbohydrate constituents between rod-shaped secretion 6 (S6) (Figure 8c). The secretions (S5 and S6) also appeared with carbohydrate constituents (Figure 8d). The transverse section of MVD showed that spermatophores with rod-shaped secretion S6 and granular secretions S7 formed a hard matrix among the spermatophores (Figure 8e). The secretion found in the MVD was granular, strongly acidophilic, and secreted by its highly columnar epithelium (secretion 7). At the end of the MVD, the fully formed spermatophores were embedded in S6 and S7 (Figure 8f).

4. Discussion

Currently, studies that have investigated the reproductive mechanism of the Blue crab are restricted to a few reports [27,38]. Besides, these studies are generally old, which merits further investigations to confirm the findings and gain new insights. In decapods, the male reproductive system of the malacostracans is found in the cephalothorax or thorax [1,39].

4.1. Morphometric Characteristics

The gonadosomatic index (GSI) of the crabs obtained in this study suggests that reproduction is likely to increase significantly in spring compared with winter and summer but similar in all to autumn. These results were contrary to the previous findings in closely related species, including Callinectes ornatus, Callinectes danae, and Callinectes bocourti collected from Sao Vicente Bay/Estuary Complex [16], where the GSI was similar across the different seasons, an indication of continuous reproduction. In addition, Sant’Anna, Turra [40] reported continuous reproduction for C. ornatus and C. danae in the Santos-Sao Vicente Estuary/Bay complex. The reason for the differences could be related to food composition and availability in different seasons. For example, Belgrad and Griffen [41] reported that the food quantity consumed had a significant influence on the reproductive effort and long-term storage of energy in C. sapidus. The seasonal changes in energy allocation for reproduction were also reported in the semi-terrestrial crab Sesarma rectum [42]. The same reasons could be said for the carapace length and width that exhibited similar trends as the GSI in this study. Furthermore, despite the total body weight of the crab showing a similar trend in this study, the difference across the seasons was not significant. The relationship, however, could be noted between GSI and total body weight, suggesting that reproduction in mature male blue crabs increases with the increase in total body weight. Similarly, Jivoff, Hines [43] reported an increase in fecundity of female blue crab with an increase in body size.

4.2. Testis and Vas Deferens

The male reproductive system of the Callinectes sapidus showed two divisions: the testis and the vas deferens similar to what was reported in the same species by Johnson [27], and
in other crab species such as *Callinectes ornatus* [44] and *Callinectes danae* [39]. The testes were lobular with highly compacted seminiferous tubules. It is believed that the spermatozoa are transported into the vas deferens through these tubules [16]. Four consecutive gonadal differentiation stages (immature, maturing, mature, and spawning) were observed for *Callinectes sapidus* in agreement with previous findings [45]. However, in some brachyuran crabs, the testes undergo only two developmental stages: the immature and mature stages based on the presence or absence of spermatophores in the distal vas deferens and ejaculatory ducts [46].

Our findings further showed that the vas deferens encases the spermatozoa in spermatophores, shielding them from desiccation and microbial infection. As reported in a previous study [43] and in other *Callinectes* species, such as *Callinectes danae* [47], and *C. ornatus* [16], the transverse parts of AVD in this study revealed the presence of spermatophores, which were irregular triangular-like shapes surrounded by two epithelial secretions. In *S. philippina* [46], spermatophore formation occurs in the distal AVD rather than at the junction to the testes, as in *C. opilio* [48]. The sperms in AVD were compacted into irregular triangular-like shapes surrounded by two epithelial secretions, and this was in agreement with previous findings in the same species [16,43]. The spermatozoa were surrounded by epithelial secretions of the AVD that consolidate the sperm mass and build the non-cellular spermatophore wall layers in the testes, where spermatophore formation starts into the vas deferens. Hinsch and Walker [48] reported that sperm maturation, spermatophore development, seminal fluid processing, and storage took place in the vas deferens in crustaceans. Therefore, the anterior part of the vas deferens is responsible for early spermatophore formation and seminal fluid development [49]. In this study, we observed that the anterior vas deferens (AVD) was divided into two distinct portions: proximal epithelium and distal epithelium, usually labeled as AVDp and AVDd, respectively. These portions have also been reported in several other studies in *C. sapidus* [16], in *C. ornatus* [44], and the majid *M. brachydactyla* [22]. According to [1], the secretions of the AVD are involved in spermatophore formation, with substance I secreted by the AVDp separating the sperm mass into small clumps. Substance II is secreted by the AVDd, consolidating the small clumps resulting in the spermatophore. This pattern has also been observed in several brachyurans as in this study. It is claimed that the second secretion was the most frequently observed secretion of the two forms [19]. Klaus and Brandis [50] proposed that mature spermatozoa join the AVDp, with each sperm cell embedded separately in an amorphous material, probably of testicular origin and homologous to the mucous smear seen in *Sundathelphusa philippina* [47]. The AVDd, on the other hand, was a highly proliferated inner epithelium that secretes the final spermatophore ground material, and vesicles produced by apocrine secretion in *S. hydrodroma* are transmitted to sperm cells, where the vesicles’ cellular components are dissolved [2]. Watanabe, Nascimento [16] reported that both the AVDp and AVDd in *Callinectes* species had the same lobulated nucleus with the epithelium filled with the rough endoplasmic reticulum, Golgi complex, and secretion vesicles between microvilli. Similar to the findings of Jivoff, Hines [43], the spermatophores were surrounded in the vasa deferentia by globules of seminal fluids. This suggests that during copulation, the spermatophores and seminal fluids are moved to the female’s seminal receptacle. Since the sperm is enclosed within the spermatophore walls, likely, they are later no longer surrounded by discrete spermatophore walls. The condition is similar to that of *Ovalipes ocellatus* at first, but as the seminal fluids are reduced, and the spermatophore wall breaks down, the contents resemble those of *Libinia emarginata*. The free sperm in the sperm masses are most likely held in the sperm masses before oviposition [51]. However, the spermatophore formation in *Callinectes* begins in the AVDp portion, caused by two separate substances secreted in the AVD. [22]. *Goniopsis cruentata* has been found to contain these two types of compounds [52]. In *Portunus pelagicus*, the AVD is a rod-shaped glycoprotein and polysaccharide chain that appears to come from the columnar epithelium of the vas deferens [19].
Moreover, the ellipsoidal pedunculate spermatophore seen in this study was complicated in the outline and sculpturing stage [49]. The spermatophore wall of C. sapidus was formed as a single acellular layer in the current study as observed in other crab species [53]. When the spermatophores are fixed and processed for electron microscopy, sectioning the materials is not difficult as it is in L. emarginata and Ovalipes ocellatus [44,51]. However, the spermatophore wall in Geryon spp. comprises two layers produced within the anterior vasa deferentia. Erkan, Tunalı [54] discovered that the spermatophores of brachyurans have a two-layered acellular wall. The spermatophore of Geryon spp. contains a large number of sperms surrounded by a thin acellular wall secreted within the anterior vasa deferentia, and the spermatophore wall is divided into two layers [19]. Simeó, Ribes [22] uncovered a translucent region between the majid crab’s two spermatophore layers and thick granules. This translucent zone is underneath the inner layer in U. uruguayensis. It is lined with thick granules that may be part of the spermatophore wall, given the heterogeneity of the wall in other decapods. As previously described, the tightly packed spermatophores in the current study leave very little space among individual spermatozoa and between spermatozoa and the spermatophore wall [55]. This study reported the spermatophores of C. sapidus in the anterior, middle, and posterior vas deferens.

Spermatophores in Scindapsus pictus were spherical, which is the most common spermatophore form among brachyurans with internal fecundation stored directly in the females’ spermathecae [54]. Klaus, Münzner [55] proposed the following mechanism in S. philippina, leading to mature spermatophores of the mucous type. Every sperm cell is embedded individually in an amorphous material, probably of testicular origin and homologous to the mucous smear found in S. hydrodroma, when mature spermatozoa join the proximal AVD. [56]. The mucous envelope that surrounds each sperm cell dissolves over time in the proximal AVD. The vesicles are transmitted to the sperm cells through apocrine secretion in S. hydrodroma [56], where the cellular components of the vesicles are dissolved. This contrasts with C. opilio, where the spermatophore content was stated to come from merocrine secretion [28,53], which was also the case in this research.

In contrast to anomuran spermatophores, the spermatophores of Portunus pelagicus are spherical or ellipsoid, non-pedunculate, and vesicular [29]. Using a scanning electron microscope, the spermatophores of Callinectes sapidus appeared ellipsoidal and pedunculate in the current study. The spermatophore diameter varied, but the spermatophores in this study were identical to those previously reported in Callinectes sapidus [27].

Similar to previous findings [43], the middle vas deferens (MVD) was the most enlarged section of the vas deferens observed in the present study. The MVD has been identified as the main storage part for the spermatophore in C. sapidus pending transfer to the PVD. [16], and in other crab species [22,57]. However, variation in the storage of spermatophore has been observed in some brachyurans among decapods. Some brachyurans have interspecific variations in terms of storing spermatophores in the PVD. [58]. The variations existing in the storage of spermatophores among different crab species could be attributed to morphological convergence [16]. The musculature in the AVD and MVD may be linked to spermatophore formation and movement since muscular contractions may separate the sperm mass [44]. Sperm transfer in Callinectes danae occurs during mating through muscular contraction, as defined by other Brachyura [59,60]. This process may be aided by the thin muscles surrounding the epithelium of the vas deferens, as discovered in this research.

On the other hand, the PVD was packed with luminar agranular substances and few ovoid spermatophores. Similar to the findings of Santos, Lima [25], the cylindrical epithelium was found in the proximal vas deferens (PVD), with loose spermatozoids in the lumen but without spermatophores. The PVD. epithelium in Libinia emarginata and Libinia dubia showed secretory activity and had an ultrastructure close to the AVD and MVD. [48]. The spermatophores in this study were found to be loosely packed in the PVD, indicating an increase in seminal fluid. This increase in the seminal fluid may be responsible for protecting and maintaining the spermatozoa [29]. The seminal fluid in
Ulmeritoides uruguayensis is secreted by epithelial cells of the vas deferens and is made up of a viscous granular matrix (seminal plasma) and electron-dense granules with a homogeneous appearance [53,61]. In the PVD region of Scyllarus chacei, it has been reported that spermatophore formation and seminal fluid development were simultaneous Hinsch and McKnight [49]. Furthermore, the shape of vas deferens lumen contraction and the deposition of acellular materials in the spermatophore layers appeared to have an influence on the spermatophore’s shape [54]. Different secretions are formed in each part of the vas deferens in brachyuran crabs [62].

4.3. Spermatogenesis

During spermatogenesis and spermiogenesis, the shape of the cellular nucleus was changed, and its size was reduced from spermatogonia to mature sperm. This decrease has also been noticed in other crabs [19,23,44]. Interestingly, however, in Maja brachydactyla, no nuclear auction was reported during spermatogenesis [22]. Although the cells were smaller in Callinectes ornatus and differed from C. danae, there was no difference between spermatogonia and primary spermatocytes [44]. The nuclear volume of C. ornatus did not shift, suggesting that the genetic material is still distributed in prophase I of meiosis, as it is in spermatogonia. The nuclear morphology of the spermatogonia and the primary and secondary spermatocytes in this study were similar to those reported in other Brachyurans [39]. The nucleus of primary spermatocytes includes standard meiotic figures, such as synaptonemal complexes in the pachytene stage during the early stages of spermatogenesis of C. sapidus. Wang, Sun [14] reported that the cytoplasm contains few mitochondria, developing ER and other membrane arrangements, as well as a nuage in agree within M. brachydactyla [21].

Nuage, concentric membrane system, and annulate lamellae are three distinct features of M. brachydactyla spermatocyte’s cytoplasm. The annulate lamellae are a network of parallel intracytoplasmic membranes found in somatic and germ cells [63]. The nuage appears as an electron-dense body during most primary spermatocyte stages, with the Diplotene stage being the most common, but the nuage appears in this study in the Zygotene stage. In the stages of leptotene and pachytene, the concentric membrane system occurs with lateral dilatations in pachytene. The annulate lamellae first appeared during Diplotene [22]. However, annulate lamellae were found during the leptotene stage in this analysis.

The basic changes that occurred during spermiogenesis were summarized using TEM as follows: (1) cellular polarization was caused by the marginalization of the nucleus, along with the development of the proacrosomal vesicle; (2) formation of a ring by the membranous system; (3) development of the operculum and perforatorium in the acrosomal vesicle; (4) nuclear surrounding of the acrosome; and (5) development of the radial arms that is consistent with [22,55]. Spermatids also undergo chromatin reorganization and packaging in three stages (St1–3). St1 is marked by the beginning of heterochromatin decondensation, with a few small heterochromatin clusters [19]. Few clumps of heterochromatin remain in St2, and the number of small, nuclear envelope-bound vesicles has increased. In comparison to the present results, Golgi complexes and mitochondria are visible. The formation of the acrosome vesicle is linked to the existence of Golgi complexes at this point [19]. The nucleus has fully decondensed at the St3 level, and a granular belt has formed as the acrosome begins to take shape [17]. The spermatid maturation sequence in C. ornatus, [44], which includes three developmental stages, is quite similar to that defined by [39] for C. danae, where the round nucleus gradually grows, and the pro-acrosomal vesicle is visible in the cytoplasm in early spermatids. Below the acrosome, the nucleus of intermediate spermatids takes on a C-shape. Late spermatids have a slender nucleus that forms a cup around the acrosome, almost fully wrapping it.

As in M. brachydactyla [22], chromatin decondensation occurred in the mid-spermatid in this study. In Cancer sp. [17] and M. brachydactyla [55], a low histone to DNA ratio and
a high degree of histone acetylation were observed. This may explain the decondensed chromatin in these species.

The cytoplasm in *M. brachydactyla* is severely reduced during spermiogenesis, decreasing to a thin band between the nucleus and the acrosome. The cytoplasmic reduction is induced by the acrosome’s production and, most likely, the release of cytoplasmic regions, which is particularly strong at the end of spermiogenesis [22].

The nucleo-cytoplasm complex is formed when the nuclear envelope disintegrates near the acrosome’s basal region during spermiogenesis, allowing chromatin to contact the cytoplasm. In *M. brachydactyla*, the nuclear envelope also produces a pentalaminar system [22]. Through spermiogenesis, the various organelles are also changed. The mitochondria in *C. sapidus* are scarce and have degenerated cristae, as defined for *M. brachydactyla* [22]. In some crabs, such as the European green crabs (*Carcinus maenas*) and freshwater crab (*Sinopotamon yangtsekiense*), the mitochondria during spermiogenesis undergo a process of aggregation and number reduction through fusion or cristae degeneration, resulting in the loss of oxidative function in certain cases [64]. In fiddler crab (*Uca tangeri*), the mitochondria are incorporated in the so-complex of the spermatozoa at the end of spermiogenesis [65].

The ultrastructure of sperm from 29 species belonging to 25 genera is currently recognized [50]. The spermatozoal acrosome of *S. philippina* is similar to that of other gecarcinucid species in terms of morphology. Furthermore, the acrosome dimensions are within the range of gecarcinucid acrosome dimensions compared to the morphologically more complex spermatozoa of the freshwater crab family. Potamidae’s uniform morphology of gecarcinucid sperm cells within this Philippine lineage can thus be verified [50]. There is no distinguishable distinction between *Callinactus sapides* sperms and *Geryon fenneri* and *G. quinquedens* sperms.

*Geryon* spp. sperms have a complex acrosome, a membranous lamellar complex, uncondensed chromatin in the nucleus, and nuclear arms or spikes, which are characteristic of brachyuran crabs. The acrosome has several compartments of varying electron density. The lamellar complex is much smaller than the extensive structure seen in the crayfishes *Cherax tenuimanus* and *Cryptococcus albidus* [49].

An electron-dense operculum, dome-shaped like in *Coenobita clypeatus* and conical like in *Birgus latro*, caps the acrosome in the present research. In contrast to *C. clypeatus* sperms, *B. latro* sperms have residual cytoplasm beyond the operculum. The subopercular zone spreads as a column posteriorly from a sub-terminal location under the operculum in both species. It is divided into two consecutive regions of differing electron density. The posterior area of the subopercular zone in *C. clypeatus* is coarsely granular instead of the finely granular appearance of the same region in *B. latro* [66]. In the thoracotremata, the operculum is located in the acrosome’s anterior portion has various morphologies [66]. According to [22], the acrosome in *M. brachydactyla* comes from the Golgi-Like Complex. We must develop a strategy to ensure the sustainable use of living marine resources and the corresponding responsibility for their management for sustainable aquaculture and fisheries development [67,68].

5. Conclusions

The morphometric study showed that carapace length was 59 ± 7.12 mm and 126 ± 18.8 mm width. The vas deferens is sectioned into anterior, middle, and posterior vas deferens. The MVD and PVD are also secretory regions, and the ultrastructure of the PVD shows that it plays an important role in changing the seminal fluid into a homogeneous fluid secretion. This is a key role during sperm transfer since this will be the first secretion inside the seminal female receptacle.

Meanwhile, the histochemical study detected two testicular secretions and six vas deferens secretions. Scanning electron microscopical study for fully formed spermatothore taken from the middle part of the vas deferens indicates that it is ellipsoidal in its outline with obvious terminal stalk. Moreover, Ultra-study showed that the testes con-
tained spermatogonia, primary spermatocyte (a) Preleptotene (b) Leptotene (c) Zygote (d) Pachytene (e) Diakinesis, Secondary spermatocytes, spermatids, and spermatozoa.

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