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Corso di Laurea Magistrale in Biologia Marina

Caratterizzazione della riproduzione del granchio blu (*Callinectes sapidus* Rathbun, 1896) nel Mar Adriatico centro-settentrionale

Characterization of blue crab (*Callinectes sapidus* Rathbun, 1896) reproduction in the northern-central Adriatic Sea

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RIASSUNTO

Questo studio si concentra sulla caratterizzazione della riproduzione del granchio blu (*Callinectes sapidus*) nel Mar Adriatico centrale, una specie aliena invasiva che ha causato danni significativi agli ecosistemi e alle attività economiche locali. L'obiettivo dello studio è comprendere le strategie riproduttive di questa specie nel Mediterraneo, confrontandole con quelle documentate nell'Atlantico, il suo habitat naturale. La raccolta dei campioni è avvenuta tra settembre e novembre 2023, con analisi istologiche delle gonadi per osservare i diversi stadi di sviluppo.

I risultati mostrano che la maggior parte delle femmine è stata catturata offshore, mentre i maschi erano più numerosi nelle zone costiere. Non ci sono state differenze significative nella biometria delle femmine, ma le femmine offshore tendevano ad avere dimensioni maggiori, probabilmente perché migrano verso il largo dopo l'accoppiamento. Anche i maschi offshore erano più grandi rispetto a quelli inshore, suggerendo che solo i maschi più robusti sono in grado di migrare. L'indice GSI (Gonadosomatic Index) era più alto nelle femmine offshore rispetto a quelle inshore, con un picco registrato a settembre, confermando un picco riproduttivo in quel mese. Gli stadi di maturazione delle gonadi variavano, indicando che le femmine possono affrontare più eventi riproduttivi durante la stagione.

Lo studio conclude che il granchio blu mostra una strategia riproduttiva complessa, con differenze significative tra gli individui che si trovano vicino alla costa e quelli in acque più profonde. La comprensione di questi meccanismi è fondamentale per sviluppare strategie efficaci per il controllo della sua diffusione e mitigare gli effetti negativi sugli ecosistemi locali e sulla pesca.

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CHAPTER I

INTRODUCTION

1.1 Biology of the blue crab Callinectes sapidus *Rathbun, 1896*

1.1.1 Taxonomy

Phylum	Arthropoda
Subphylum	Crustacea
Class	Malacostraca
Subclass	Eumalacostraca
Superorder	Eucarida
Order	Decapoda
Suborder	Pleocyemata
Infraorder	Brachyura
Superfamily	Portunoidea
Family	Portunidae
Genus	*Callinectes*
Species	*Callinectes sapidus*

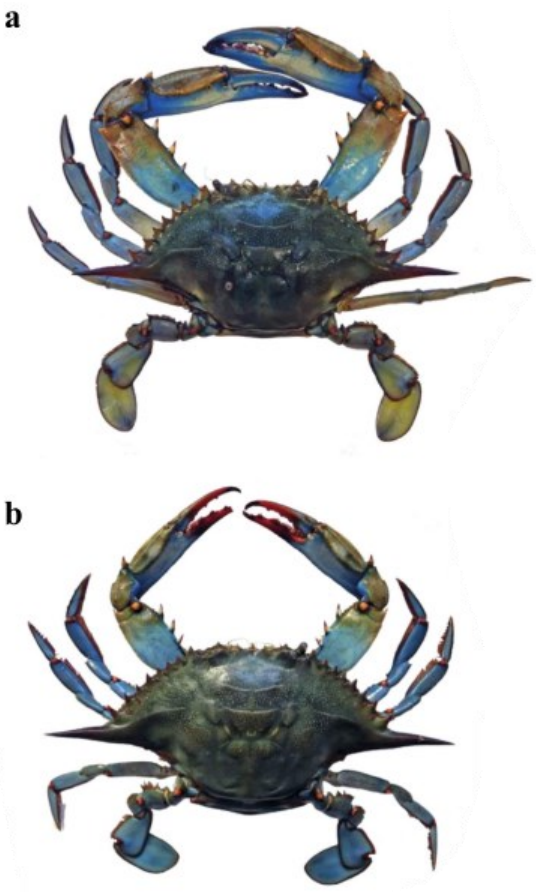


Figure 1. Taxonomy classification and adult specimens of *Callinectes sapidus*, adult male (a) and female (b).

Callinectes sapidus Rathbun, 1896 (**Fig. 1**) is also known as "blue crab", due to the blue color that characterizes its upper surface and especially its claws. It is a crustacean belonging to the Portunidae family, which can be distinguished by the presence of flattened posterior legs that they use to swim; in fact, they

are called swimming crabs (Churchill et al., 1919). Another distinction within the family can be made by looking at the abdomen, or apron, in fact all males that have it T-shaped belong to the genus Callinectes (Millikin et al., 1984).

Callinectes sapidus derived from Latin: *Calli*, beautiful; *nectes*, swimmer; and *sapidus*, savory. So, the literal translation of this species' name is "beautiful, savory swimmer" (Guillory et al., 1996). This could be a result of both the previously mentioned characteristic of the family and the fact that this species is widely consumed as food, in fact it supports the commercial and recreational fisheries along the Atlantic and Gulf of Mexico coasts of the United States (Millikin et al., 1984).

1.1.2 Morphology

The taxon of Malacostraca, to which the blue crab belongs, presents a body structure characterized by three parts: head or cephalon, thorax or pereon (with the pereopods, associated appendages) and abdomen or pleon (with pleopods, associated appendages). Every part has different numbers of somites, in the blue crab there are 5 somites in the head, 8 thoracic and 6 abdominal; these segments could be fused or not, in this species the head somites and the thoracic ones are fused to form the cephalothorax. The presence of the skeleton does not allow the animal to grow, so it must be renewed periodically through the molt (Kennedy et al., 2007).

The swimming ability in the blue crabs is favored by both the presence of the swimming legs, the modified posterior legs, and a body shape that is twice wider than long and dorsoventrally flattened. These characteristics enhance hydrodynamics, facilitating efficient movement through water (Kennedy et al., 2007).

As regards measurements, the carapace width (CW) can be up to 20,9 cm in males and 20,4 cm in females, with respectively carapace length of 9,1 cm and 7,5 cm (FAO, 2024).

The anterior margin of the carapace comprises a frontal region or medial, between the eyes, and lateral regions or anterolateral; all these regions are studded with teeth (**Fig. 2a**). In the middle part of the frontal region there is the epistomial spine, on either side of which are the first antennae, then second antennae and finally the eyes (Kennedy et al., 2007). The inner part between the orbits has two broad, either obtuse or acuminate, triangular frontal teeth, while laterally the carapace presents nine anterolateral teeth, the larger one on the lateral corner is called lateral spine (FAO, 2024; Hill et al., 1989; Millikin et al., 1984). Proceeding posteriorly, the carapace narrows and has no other teeth. Looking dorsally, this is characterized by a series of dentate ridges and grooves which divide the carapace surface into smaller regions, whose names are indicative of the organs below (Kennedy et al., 2007).

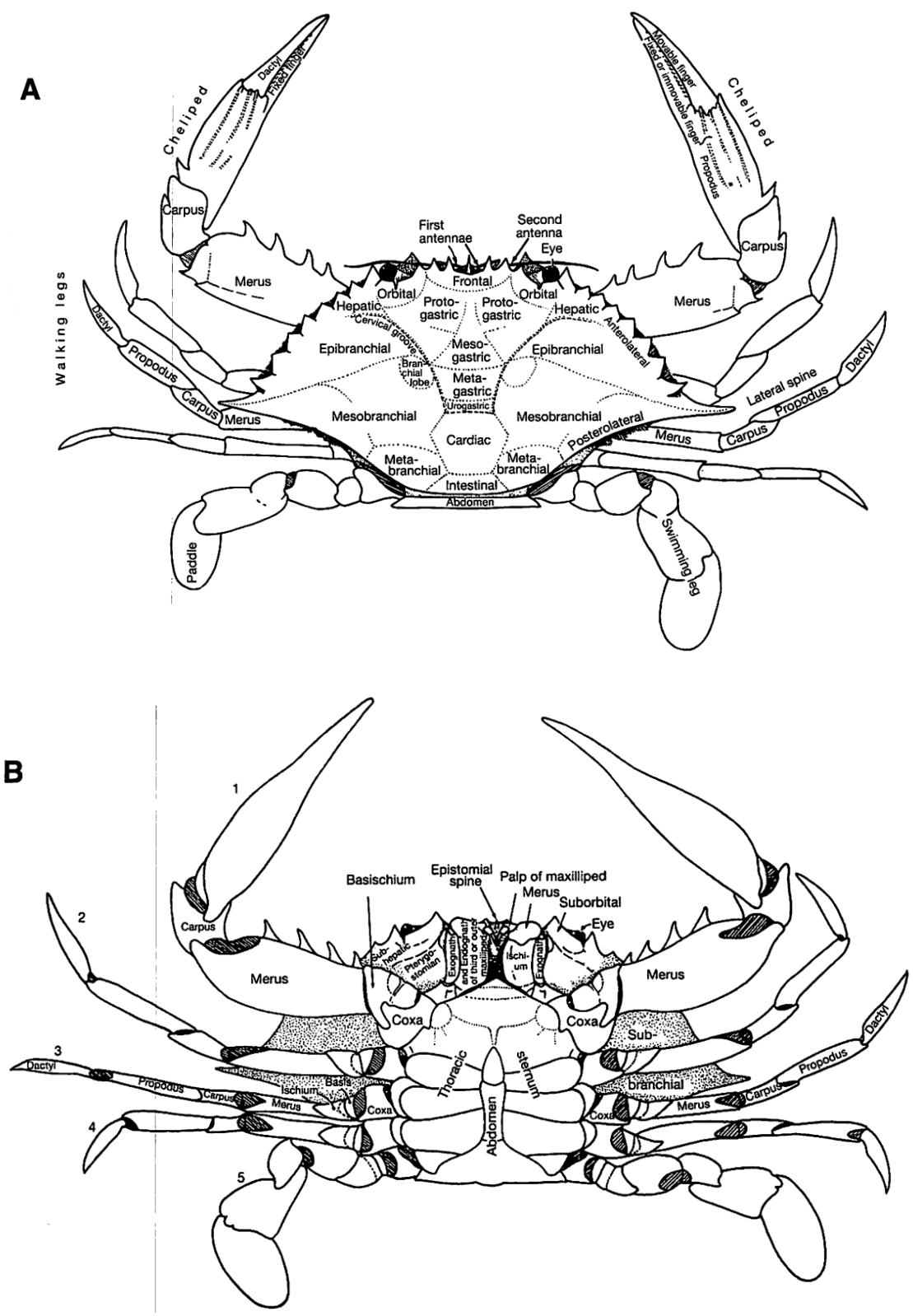


Figure 2. (A) Dorsal and (B) ventral view of a mature male blue crab, the pereopods are numbered from 1 to 5. From Kennedy (2007).

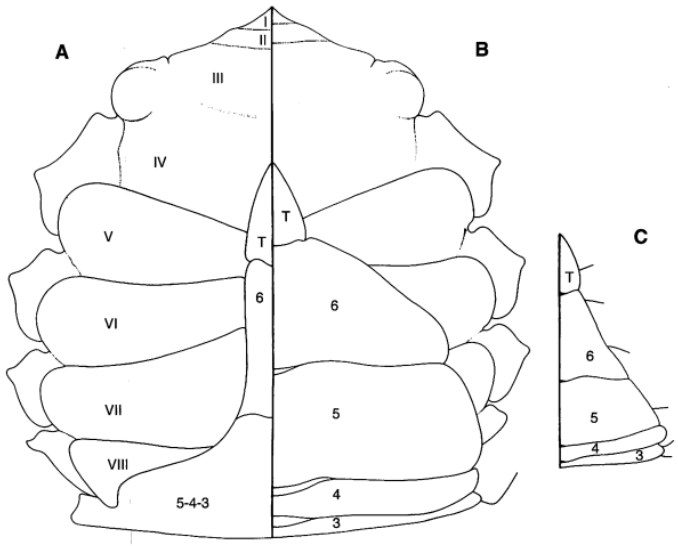


Figure 3. Composite ventral view of thoracic sternites (roman numerals), abdomen (arabic numerals), and telson [T] in situ: (A) Mature male. (B) Mature female. (C) Immature female. From Williams (1974).

The ventral portion is also divided into distinct regions (**Fig. 2b**). Moreover, viewing ventrally (**Fig. 3**) it is possible to determine the sex of the specimens, in fact males and females are recognizable by the shape of the abdomen, or apron (Kennedy et al., 2007).

In the male the shape of the abdomen doesn't change throughout its life and has a characteristic T-shape. Instead, in the female presents different shapes before and after reaching sexual maturity, as it plays an important role in the retention of embryos. It changes from an initial triangular shape to a broader, wider one (Brown, 2009). Usually the abdomen, in both sexes, is closed under the body in a depression of the thorax; there is a system that locks the abdomen in that position in both males and immature females. This system can be unlocked in males for copulation while in females it is no longer functional when they reach

sexual maturity, to allow copulation and the expansion of the embryonic mass (Kennedy et al., 2007).

All the species of the genus Callinectes have the chelipeds modified into a major chela and a minor chela, respectively crusher, usually on the right, and cutter, usually on the left; thus, the species are defined heterochelic and heterodont. The chelipeds are strong with irregular cutting teeth and have granular ridges. The merus presents three very strong inner spines with increasing size (FAO, 2024; Kennedy et al. 2007).

In the blue crab the color of the dorsal carapace and the cheliped may vary, the most common hues are shades of gray, blue and brownish green (**Fig. 4A**). Ventrally they have an off-white tint with some shade of yellow and pink (**Fig. 4B**). In the leg articulations they have orange tubercles as the legs are mostly blue and white with lesser amounts of red and brownish green. In the male the propodi of the chelae are blue with red tips while in the mature female they are orange with purple tips (**Fig. 4C**) (Guillory et al., 1996). The carapace is slightly convex and smooth, except for small tubercles on mesobranchial, posterior slope of cardiac, and anterior portion (Millikin et al., 1984).

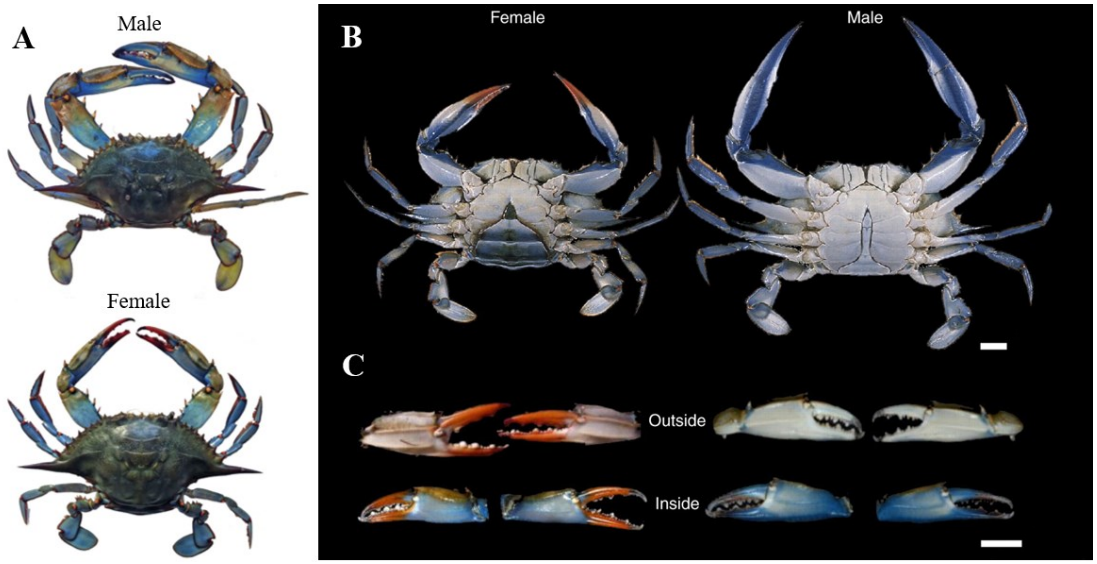


Figure 4. Dorsal view of *Callinectes sapidus* (A), ventral view (B) and propodi of the chelae (C).

The endoskeleton of crustaceans is formed by several cuticular infoldings, or apodemes. The function of apodemes is to support the skeleton, and in some cases, they also provide a surface for muscle attachment. There are also other thin structures that function like tendons to attach muscles to the skeleton (Kennedy et al., 2007).

For what concerns the body organs (**Fig. 5**), removing the dorsal carapace, they are covered by a pigmented hypodermis, whose broke reveal the visceral cavity. Barely behind the frontal region of the carapace is located the foregut, it is characterized by white ossicles, in the dorsal part, and by a series of visible alimentary muscles (Kennedy et al., 2007). The gills are located on both sides of the body and taper from the outside of the carapace inwards, these are arranged within branchial chambers that protect them, occupying most of the

area behind the glands of the midgut and lateral to the heart (Kennedy et al., 2007). Dorsally to the foregut occurs the bladder but, whit the removal of the hypodermis, it is generally broken. In immature blue crabs the anterior and lateral cephalothorax is full of the midgut glands, organized in small lobules within larger lobes of a color ranging from yellow to green. Behind the heart and beneath the connective tissue are placed the midgut, the midgut ceca, and the posterior part of the midgut glands; in the male the testes are highly convoluted and posterolateral to the foregut lies the vas deferens that can fill the space between the branchial chamber and the foregut (Kennedy et al., 2007).

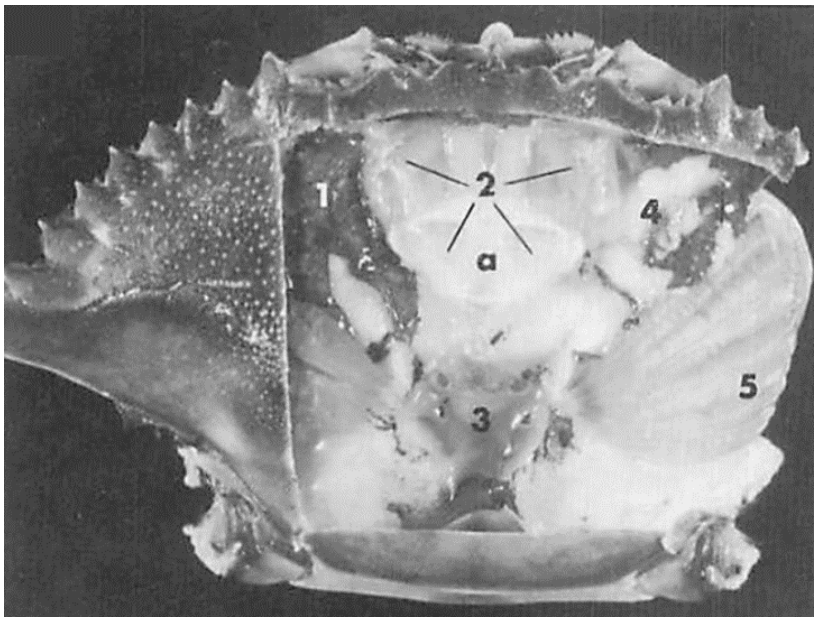


Figure 5. View of blue crab's visceral cavity after most of the carapace has been removed. Hypodermis [1]; cardiac foregut [2]; heart [3]; intermingled midgut glands (white lobes) and convoluted testes [4]; gills [5]; hemopoietic tissue. From Kennedy (2007).

The ovaries have a typical H shape, thanks to an intermediated connection positioned at the level of the medial stomach. The female gonads lie dorsal to the midgut glands and expand on both sides of the anterior margin of the body cavity in the dorsal part of the carapace, mingling with the hepatopancreas, then descend towards the cardiac stomach and continue covering the posterior stomach (Browm, 2009).

1.1.3 Habitat and distribution

Callinectes sapidus is native to the western coasts of the Atlantic Ocean and its range extends from southern New England to Argentina. In the 19th century, it was introduced to Europe via ballast waters and subsequently its distribution rapidly expanded into the Mediterranean Sea and neighboring waters, and it is now considered an invasive alien species; it also reached the Asian continent through ballast waters (**Fig. 6**). The blue crab is an important predator that inhabits coastal habitats such as estuaries, lagoons, bays, and others. It is euryhaline and eurythermal (Epifanio, 2019; FAO, 2024; Mancinelli et al., 2017a). The Chesapeake Bay has the largest semi-confined blue crab spawning area, multiple nursery areas and optimal conditions for this species on the entire west coast of the Atlantic Ocean. In fact, the crab represents an important source of fisheries in this bay; almost half of the commercial landings of blue crab in the US occur there (Harris, 1982).

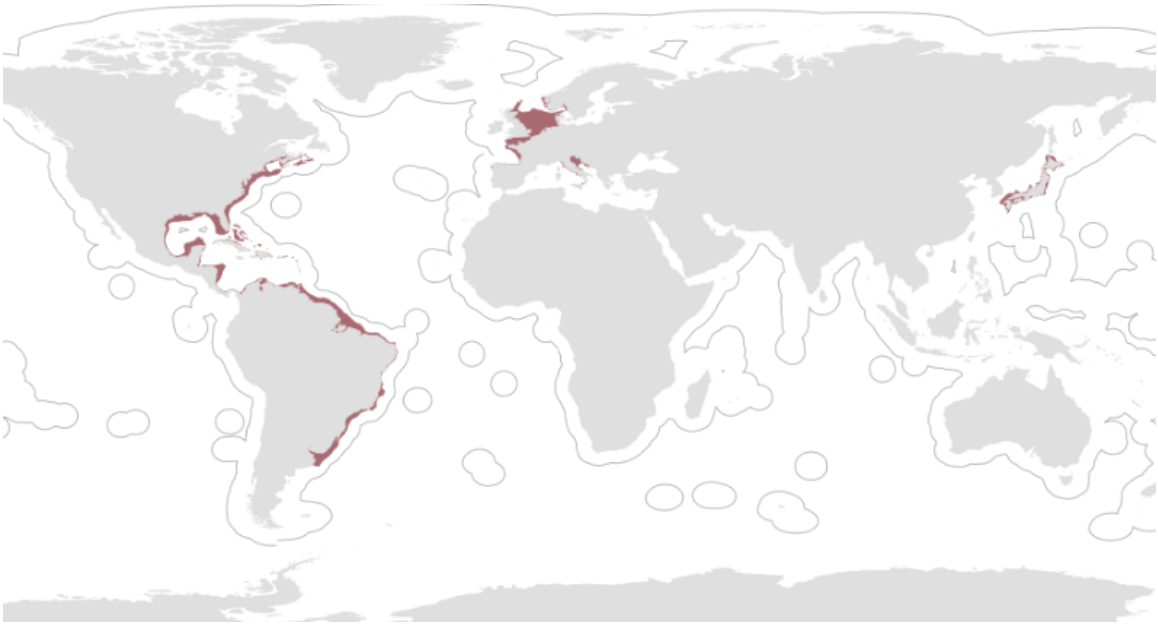


Figure 6. Distribution of *Callinectes sapidus*. From FAO (2024).

This species is distributed at depths between 0 and 90 m on muddy and sandy bottoms; generally, the smallest crabs are found near the coast and can be found in water up to a few centimeters deep, while the adults are found in deeper waters (Churchill et al., 1919; FAO, 2024).

Callinectes sapidus during its life cycle inhabit different types of habitats. High oceanic salinities and at least 20°C are required by the larval stages to develop; the next stage, the megalopa, returns to shallow estuarine habitats to settle in nursery areas. Seagrass beds and macroalgal patches are structured bottom habitats used by blue crab as nursery areas for megalopae; they guarantee substrata for their settlement, shelter from the predator and with food resources. In each nursery area, to reduce cannibalism events, there are usually a few

distinct cohorts of juveniles per spawning season (Epifanio, 2019; Kennedy et al., 2007). In the western Atlantic Ocean, there are at least four seagrass species used as nursery habitats: *Zostera marina*, *Halodule wrightii*, *Thalassia testudinum*, and *Ruppia maritima* (Epifanio, 2019). In seagrass beds, blue crabs' megalopae reach an abundance of more than 100 crabs per m^2, and when seagrasses are not present, macroalgae patches play a key role; in fact, beds of *Ulva lactuca* and *Gracilaria spp*., green and red algae respectively, were observed to host around 100 crabs per m^2 (Epifanio, 2019). Once that the crabs reach a carapace width greater than 15 mm the shelter value of nursery areas is reduced and the predation rate decreases, this allows them to leave these areas, usually when they are 20-30 mm in size; at this point they move to less sheltered and structured bottoms also to maximize their otherwise limited growth in such a dense habitat (Epifanio, 2019; Kennedy et al., 2007). Other habitats used as nursery areas are salt marshes and some non-vegetated habitats such as oyster reefs and coarse woody debris in areas close to vegetated coastlines; however, abundance of crabs in these habitats is lower than those previously described (Epifanio, 2019).

After leaving the nursery areas, depending on size, the individuals distribute themselves in different areas. Juveniles with a 30 - 70 mm CW move along the coast in shallow waters, taking refuge in woody debris. While crabs with >100

mm CW located in deeper waters of unstructured soft-bottom habitats located in canals and estuarine basins (Kennedy et al., 2007). Adult blue crabs are typically of soft bottom habitats at >1m depth; in the summer adult males and females feed in subestuaries, while, i.e. in Chesapeake Bay main stem, mature female during the spawning season in summer and during fall migration, and males and females during the burial over-winter, used soft bottom habitats at major depths (>10m) (Kennedy et al., 2007).

1.1.4 Feeding habits

The larvae of *Callinectes sapidus* are filter feeders and feed on planktonic organisms, both phytoplanktonic and zooplanktonic; in fact, it was seen that a diet based only on phytoplankton led to a lack of protein, preventing a successful molt. The planktonic preys must have shape, dimension and the external covering that makes them easy to catch and consume (Guillory et al., 1996).

The blue crab's diet has been studied in detail in the Atlantic Ocean, and particularly in Chesapeake Bay; here is a food web of blue crab outlined following studies in this bay (**Fig. 7**).

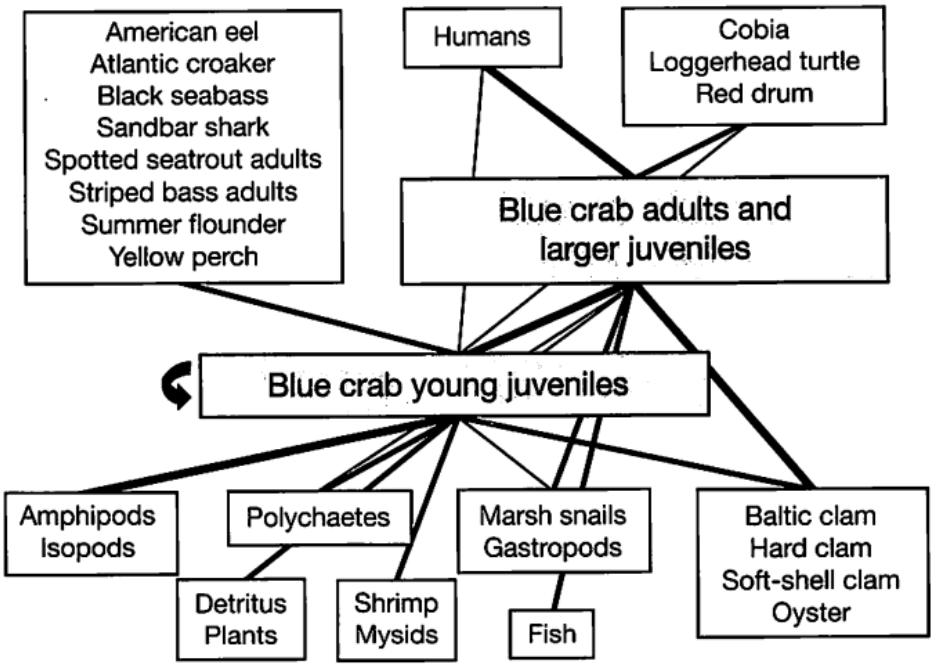


Figure 7. Food web of the blue crab in Chesapeake Bay, illustrating only those linkages with the blue crab as either predator or prey, and with emphasis on young juveniles. The compartment for blue crab young juveniles is specific to juveniles. From Kennedy (2007).

This is a complex food web in which several predators have been identified for juveniles, while only few species for the adults such as the cobia, loggerhead turtle, the red drum, and humans (Kennedy et al., 2007). The juvenile and adult blue crab has been defined as an opportunistic benthic detritivore, cannibal, primary carnivore, omnivore, and general scavenger (Guillory et al., 1996).

Juveniles and adults show different diets with some points in common. The juveniles feed mainly on benthic infauna and epifaunal invertebrates, while the typical prey for the adults are the bivalves (**Fig. 8**) (Kennedy et al., 2007).

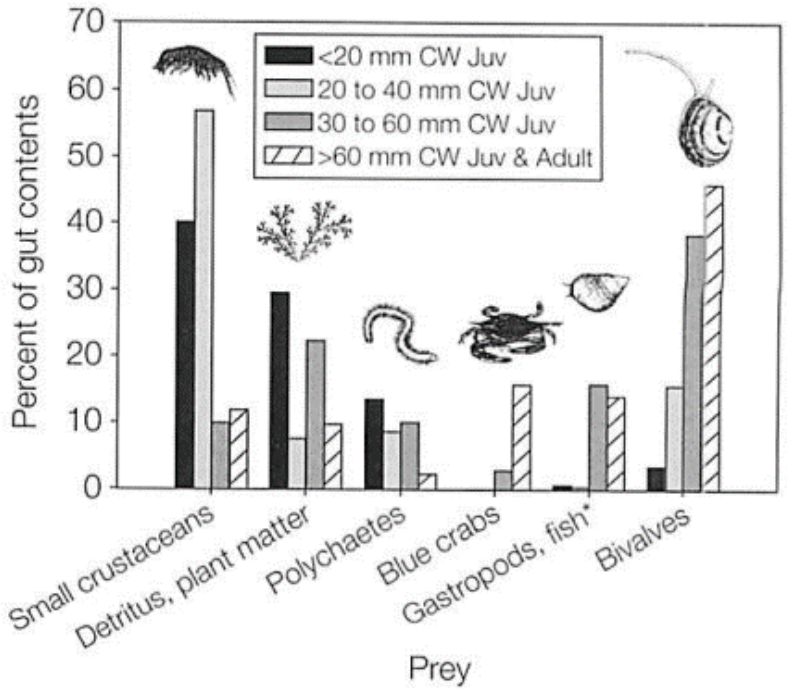


Figure 8. Comparison of blue crab gut contents from a study of small juveniles (<20 mm CW) and larger young juveniles (20-40 mm CW) with those of larger juveniles and adults (Table I), miscellaneous prey. From Kennedy (2007).

In addition to the great consumption of bivalves, adults' diet is distinguished from juveniles' one by higher rates of cannibalism, which develops from individuals >40 mm CW; while the common preys are small crustaceans, like amphipods and isopods, gastropods, polychaetes, crabs, shrimp, fish remains, detritus, plant matter and bivalves (Kennedy et al., 2007). However, the choice of the preys depends on their availability and necessarily on the type of habitat (Kennedy et al., 2007).

1.2 Life cycle

Reproduction in blue crabs involves a complex sequence of events. It begins with an extensive courtship process, followed by copulation. Once copulation occurs, the female stores the sperm, allowing for internal fertilization of her eggs later (Epifanio, 2019). Mating primarily takes place in the oligohaline and mesohaline regions of estuaries. Following mating, the fertilized female crabs migrate to the lower parts of the estuary and into the adjacent coastal ocean. Here, the females brood their eggs externally until they hatch (Epifanio, 2019).

Embryonic development is influenced by environmental conditions and lasts between 12 and 15 days at a temperature of 28°C and a salinity of 30. While, at cooler temperatures, such as 26°C, development takes longer, typically between 14 and 17 days. The color of the brood changes during development, starting yellow to orange within 1 to 7 days after extrusion, and then turning brown to black from 8 to 15 days after extrusion (Kennedy et al., 2007). Embryo loss during development is a widespread issue among crustaceans. Research indicates that about 30% of species show a reduction in brood size during this period. The degree of this loss varies greatly, ranging from 0 to 69% in crayfish and from 0 to 100% in other crustaceans (Kennedy et al., 2007). Various factors contribute to embryo loss, including poor adhesion to the pleopods, mechanical damage, disease, parasites, and predation. Despite the

prevalence of this phenomenon, detailed data on embryo mortality in blue crabs is sparse, highlighting the necessity for more focused research (Kennedy et al., 2007).

In blue crabs, larval release is synchronized through an interplay of two endogenous biological clocks: one operating on a circatidal (tide-based) cycle and the other on a circadian (daily) cycle. These rhythms are influenced by environmental cues such as salinity and light (photoperiod) (Kennedy et al., 2007). The circatidal rhythm dictates that larvae are typically released and carried seaward during morning ebb tides, reducing their exposure to planktivorous predators from nearshore shoals (Kennedy et al., 2007). However, more recent studies on other species of subtidal crab, demonstrate that ovigerous females influence the circadian rhythm in hatching embryos through water-soluble chemical signal (Epifanio, 2019).

Hatching begins when the embryo absorbs water, initiating osmotic process that leads to the rupture of the outer embryonic membrane (chorion), and with the release of pheromone that stimulate simultaneous hatching events. Subsequently, larval movements rupture the inner membrane, releasing the prezoeal larvae (Epifanio, 2019; Kennedy et al., 2007). Ovigerous females exhibit hatching behavior characterized by sustained flexing of the abdomen, facilitating larval release. Hatching occurs at the mouth of estuaries and in the

nearby coastal waters. All eggs from neighboring females are released around early morning high tide, making hatching a brief, synchronized event (Epifanio, 2019; Kennedy et al., 2007).

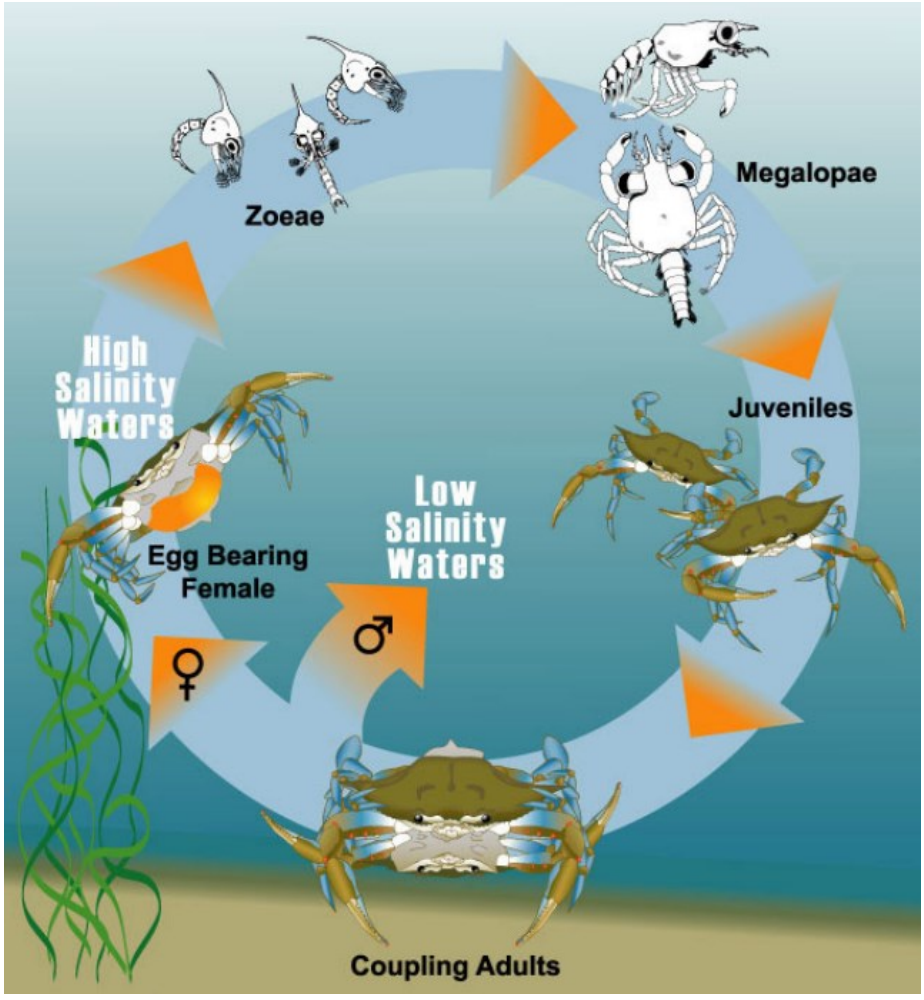


Figure 9. *Callinectes sapidus* life cycle: from zoeae to adults. From Chenery (2002).

Blue crab larval development spans several stages (**Fig. 9**): one prezoeal stage, seven zoeal stages, and a single megalopa stage (Epifanio, 2019). The molt from prezoeal to the first zoeal stage occurs within 3 minutes after release, which explains their absence in earlier research. Following larval release,

reliable indicators of recent spawning events are provided by both the inner and outer embryonic membranes, which remain attached to the female's pleopod setae for several days (Kennedy et al., 2007).

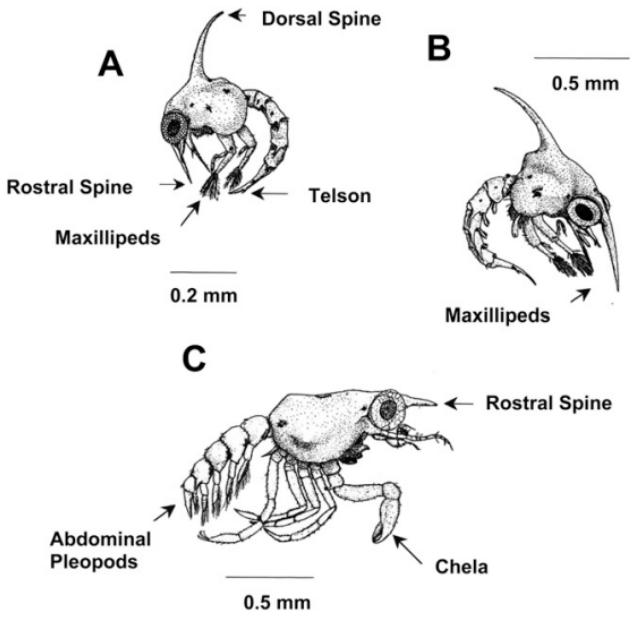


Figure 10. External anatomy of representative larval stages of *C. sapidus*. (A) Early zoeal stage. (B) Advanced zoeal stage. (C) Megalopal stage.

Under favorable conditions, zoeae development takes 3 to 4 weeks, while the duration of the megalopa stage varies significantly due to a range of chemical and physical factors. Zoeae (**Fig. 10A, B**) are able swimmers, which allows them to maintain a high position in the water column (Epifanio, 2019). Zoeae development take place in the open coastal ocean, with recruitment to juvenile populations depending on the transport of megalopae back to estuarine environments. Megalopae (**Fig. 10C**) exhibit robust swimming abilities and,

due to rhythmic vertical migrations, the upstream transport within estuaries is facilitated (Epifanio, 2019). Settlement of megalopae occurs in habitats such as submerged aquatic vegetation and oyster reefs, where metamorphosis into juveniles occurs (Chenery, 2002).

Upon metamorphosis, newly formed juveniles possess carapaces a few millimeters wide and inhabit juvenile habitats for several months, gradually reaching widths of 20–30 mm (Epifanio, 2019). The entire life cycle, from the juvenile stage onward, is confined to the estuarine environment. Growth is rapid during this phase, typically culminating in maturity within one to two years (Chenery, 2002).

Different species address the challenge of growth with rigid external skeletons in distinct ways. Clams and sea urchins achieve growth by simultaneously enlarging their hard shells and soft inner tissues (Kennedy et al., 2007). In contrast, crustaceans such as *Callinectes sapidus* employ a different strategy: they undergo molting, shedding their old shell, developing a larger replacement, and then expanding their internal tissues to fit the new shell (Kennedy et al., 2007). This molting process makes crustaceans vulnerable immediately after molting, limiting their ability to move effectively, eat or defend themselves, thus making them easy targets for predators. However, the

enhanced mobility afforded by molting may represent a trade-off compared to the relatively static nature of clams and sea urchins (Kennedy et al., 2007).

1.3 Reproductive strategy

1.3.1 Reproduction in Brachyura

Brachyuran crabs exhibit sexual dimorphism and are gonochoristic crustaceans, meaning they have separate sexes and do not undergo sex changes. This dimorphism is evident in the shape of the pleon (abdomen), its appendages, and the location of the gonopores (McLay et al., 2015).

In males, the first two pairs of pleopods are modified to form gonopods, which are specialized for insemination. The differences extend to the chelae as well, with males possessing significantly larger chelae compared to females. These enlarged chelae play a crucial role in male-male competition for mating rights (McLay et al., 2015). Females, on the other hand, typically have four pairs of biramous pleopods located on pleonites 2-5, which are utilized to carry fertilized eggs. Brachyurans, members of Pleocyemata, display this unique reproductive behavior, in which females carry cluster of eggs attached to their pleopods under the pleon until the larvae hatch, rather than spreading them directly into open water as seen in non-pleocyemate crustaceans (McLay et al., 2015). Upon reaching maturity, the female's pleon expands considerably in width, which increases her capacity to carry and protect eggs. This broader

pleon is a key adaptation for reproductive success, allowing females to support a larger number of developing eggs (McLay et al., 2015).

Reproduction in Brachyura involves intricate life history traits, sophisticated reproductive strategies, and sometimes particular behaviors for attracting mates and mating. In males, the reproductive system features paired testes located in the thorax, which open through gonopores in the eighth thoracic somite. The vas deferens extends to transport spermatophores to the base of the gonopods, which then deliver them to the female's spermathecae (McLay et al., 2015).

In females, the reproductive system comprises paired ovaries that produce eggs. These eggs are carried by the oviducts either directly to the coxal gonopores in podotremes or to the seminal receptacles in eubrachyurans. In eubrachyurans, fertilization takes place inside the seminal receptacles within the body, whereas in podotremes, it occurs externally (McLay et al., 2015).

Typically, females provide parental care, significantly increasing the survival of early instars through the shelter they offer. This behavior has been observed in brachyuran species across various habitats, indicating that it has independently evolved multiple times. Juveniles are not protected by male crabs, likely due to the uncertainty of paternity. Instead, various sperm-guarding tactics are employed by males, including pre- or post-copulatory guarding of the female (McLAy et al., 2015).

Elaborate behaviors are performed by ovigerous females to preserve their broods, which require significant energy. Embryos are groomed by the females using their chelae and other pereiopods to keep them clean and free from detritus and pathogenic epibionts. The broods are also ventilated by the females through pleonal flapping, which exposes the eggs to water flow and aids in oxygenation. Grooming and aeration are interconnected practices, as the embryos compete with epibionts for oxygen (McLay et al., 2015).

A critical aspect of maternal care is the release of larvae at the optimal time, a process stimulated by the embryos themselves as they near hatching. Researchers have observed the presence of tripeptides in *Rhithropanopeus harrisii* (Panopeidae), released from the eggs, which stimulate pleonal activity and promote hatching (McLay et al., 2015). Similarly, compounds triggering hatching have been observed in *Callinectes sapidus* throughout all stages of embryo development, with late-stage compounds eliciting the strongest response. It is possible that these mechanisms are the same as those observed in parental care during embryonic development (McLay et al., 2015).

1.3.2 Reproduction in *Callinectes sapidus*

The reproduction of *Callinectes sapidus* consists of three phases: precopulatory guarding, copulation and postcopulatory guarding (**Fig. 11**) (Epifanio, 2019). Before the first stage courtship occurs, this is based on the release of

pheromones by the mature male and by the prepubertal female; thus, the pair formation is promoted by chemical cues but also by visual signals, which, according to a recent study, may be sufficient alone in stimulating courtship (**Fig. 11A**) (Epifanio, 2019). Once the couple is formed, the precopulatory guarding begins; for several days, the male carries the female under his abdomen until her final molt (**Fig. 11B)** (Epifanio, 2019). Females reach sexual maturity at a size of about 130 – 139 mm, after 17-19 molts, with the last one occurring before the single mating, known as the pubertal molt (**Fig. 11C**). During this terminal molt the female abdomen undergoes significant morphological modification, changing from triangular shape to semicircular and increasing setae on pleopods, to ensure the surface area necessary for the embryos to be brood. Instead, males may experience another three to four molts even after reaching sexual maturity (Harris, 1982; Kennedy et al., 2007).

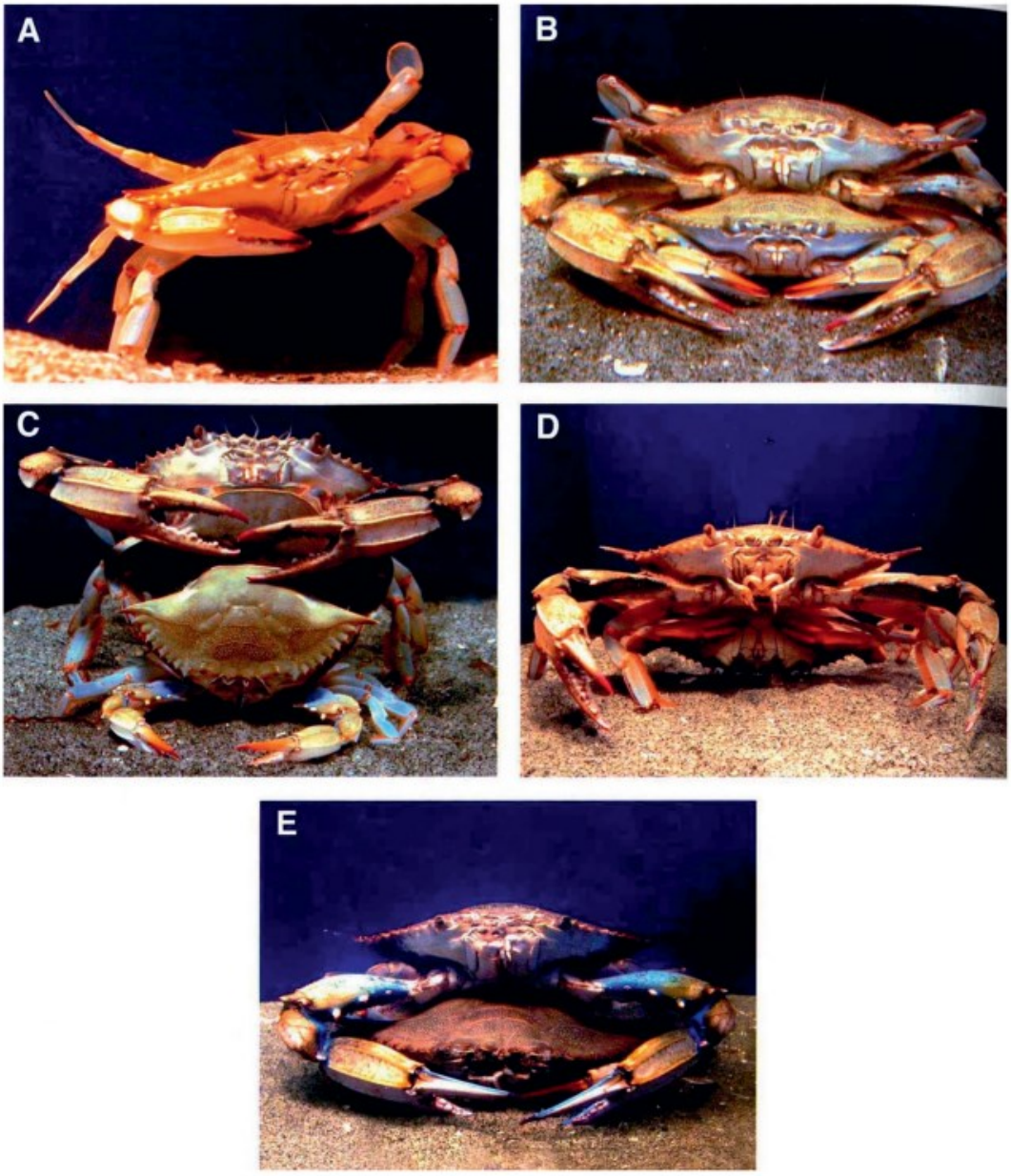


Figure 11. Mating behavior in the blue crab *C. sapidus*. (A) Male mating display. (B) Precopulatory guarding. (C) Female undergoing pubertal molt. (D) Copulation. (E) Postcopulatory guarding. From Epifanio (2019).

Copulation occurs right after the female's pubertal molt, when she is in the soft-shelled condition (**Fig. 11D**). Once her integument becomes calcified, she

becomes incapable of undergoing another copulation. In contrast, the male is in the hardened form, and he is promiscuous (Epifanio, 2019; Harris, 1982).

Copulation involves the transfer of the ejaculate, through the male reproductive structures, into the female. The ejaculate consists of seminal fluid and spermatophores, which are packets of sperm, and passes via the vas deferens, the penes, the second pleopods, and the first pleopods, by which it is introduced into the female through a pair of genital pores located on her abdomen (**Fig. 12A, B**) (Epifanio, 2019).

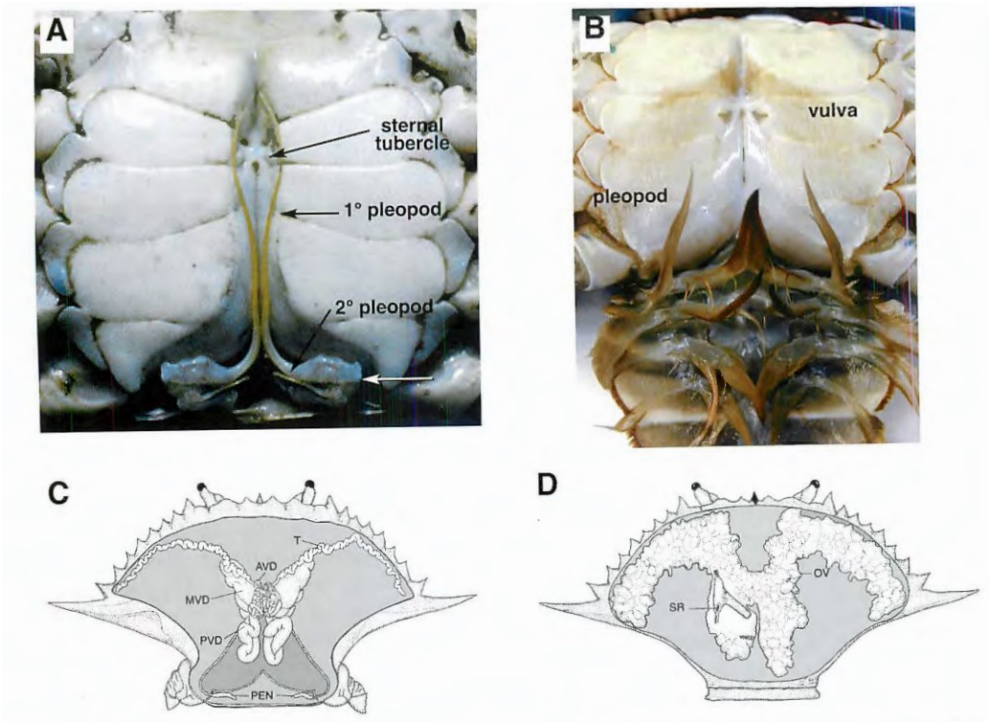


Figure 12. (A) External anatomy of male reproductive system, sternal tubercle, primary [1°] pleopod, and secondary [2°] pleopod. (B) External anatomy of female reproductive system. (C) Internal anatomy of male reproductive system - testis [T], penes [PEN], anterior vasa deferentia [AVD], middle vasa deferentia [MVD], posterior vasa deferentia [PVD]. (D) Internal anatomy of female reproductive system - ovary [OV], seminal receptacle [SR] or spermatheca. From Kennedy (2007).

The male reproductive system's external portion is located beneath the abdomen and comprises three paired structures: the penes, secondary pleopods, and primary pleopods. Both the secondary and primary pleopods feature an internal cavity, known as the ejaculatory duct, which is encased in a hard external cuticle (Kennedy et al., 2007).

Through the vaginal ducts, it reaches the seminal receptacles or spermathecae, which are paired structures dedicated to sperm storage. During copulation, the oocytes are still undifferentiated; therefore, the sperm is collected in this storage site and used later for egg fertilization (Epifanio, 2019). In the last phase, the post copulatory guarding, the male checks the female until her integument is completely calcified (**Fig. 11E**). This series of behaviors server to protect vulnerable females from predators and to guarantee the exclusive access to the male (Epifanio, 2019).

After mating, there is a variable period required for eggs maturation, ranging from 2 to 9 months. The development of the ovary is particularly influenced by environmental factors, especially temperature, where at higher temperatures, development is faster (Harris, 1982). However, time of mating also significantly influences the development of the ovary. Mating in late spring or early summer allows the ovary to develop fully before the end of summer, preparing it for egg extrusion. In contrast, mating later in the summer or early

fall delays ovarian development until the following year, resulting in egg extrusion occurring from late spring to mid-summer (Harris, 1982).

Fertilization of eggs occurs in the spermatheca; when the eggs mature in the ovary, they reach the seminal receptacles through the vaginal ducts. After fertilization, the eggs are released through the genital pores, reaching the ventral surface of the abdomen where embryonic development will occur (Epifanio, 2019). Until hatching, the embryos remain attached to modified abdominal appendages called pleopods (**Fig. 13**) (Harris, 1982).

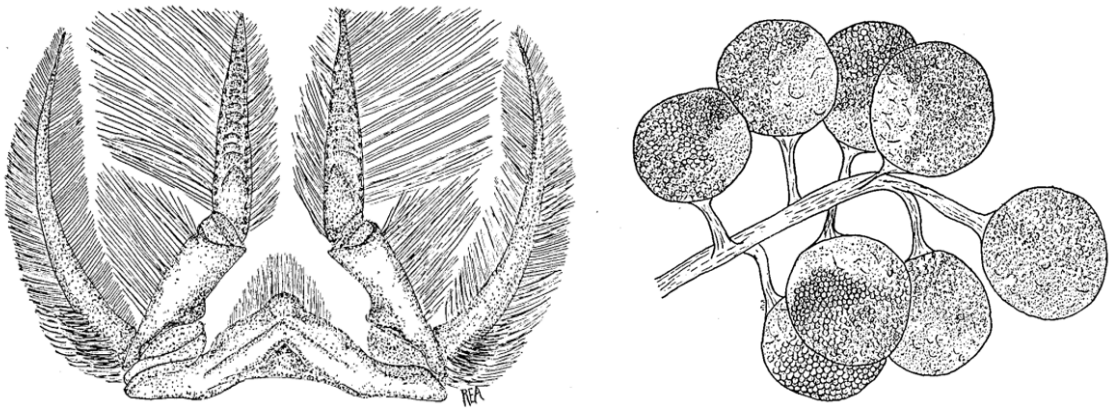


Figure 13. (A) Pleopod from abdomen of female to which eggs become attached. (B) Eggs attached to hairs of pleopod. From Newcombe (1945).

The embryos undergo a color change, from orange to black; initially, the embryos are orange due to the presence of yolk, but just before hatching, once the yolk is consumed and the eyes start to appear, the mass of embryos appears black (**Fig. 14**) (Harris, 1982). Females deter macrofaunal predators with their

aggressive behavior, complete calcification, and size. Additionally, females may flex their abdomen or groom the masses of embryos with their walking legs, ensuring aeration and cleanliness from parasites; however, the developing embryos mass remains vulnerable to fungal infection or infestation by nemertean worms, which are known to prey on them (Epifanio, 2019).

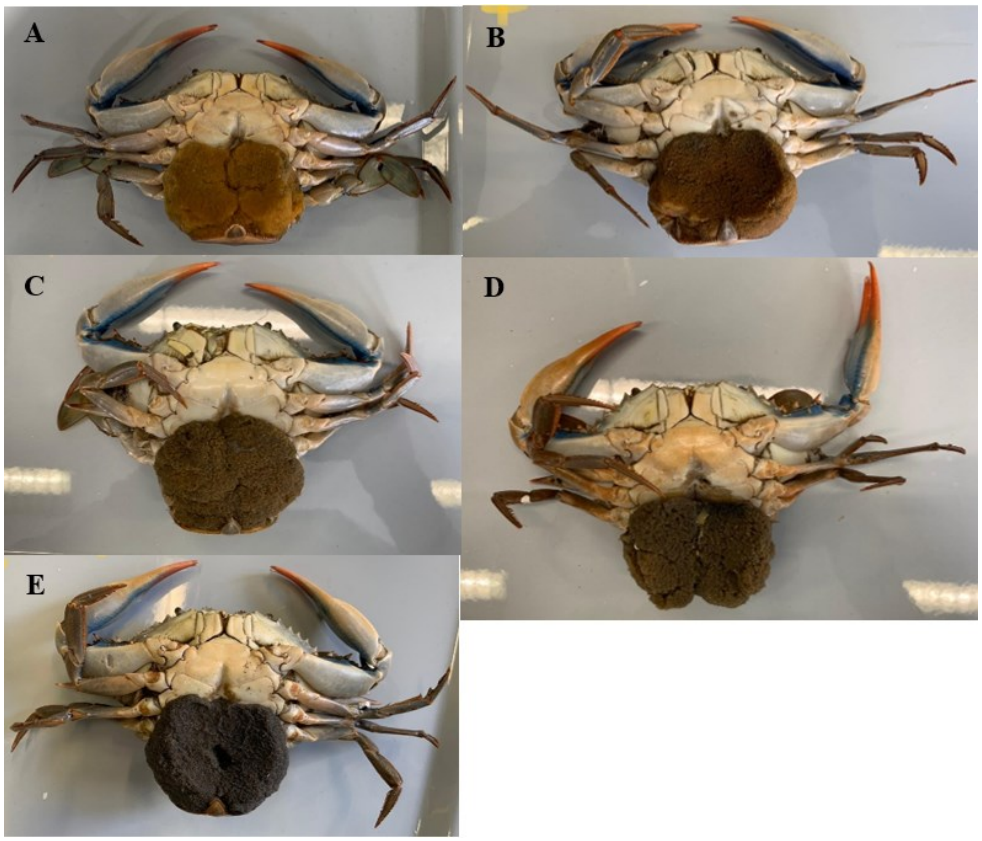


Figure 14. Five female crabs with embryos at different stages of maturation, ranging from orange to black, showing the gradual yolk consumption during development. (A) ♀70, (B) ♀71, (C) ♀72, (D) ♀73, (E) ♀74.

Anyway, blue crabs' high fecundity compensates for potential embryo losses, in fact a typical female can release from three quarters of a million to 2 million eggs each spawning (Epifanio, 2019; Harris, 1982). The female can produce a

vast number of eggs due to the small size of each individual egg, the large abdominal area designated for embryos placement, and the significant internal space available for ovarian development (Epifanio, 2019).

Between one spawning event and another, the ovary can regenerate rapidly. The spawning period in Chesapeake Bay typically spans from May to September, with a major peak between June and August (Harris, 1982). Females that release embryos in late spring can spawn again later in that same year, as well as the following spring. A female may experience three or more spawning events in her lifetime, with a decreasing number of embryos in subsequent broods (Epifanio, 2019; Harris, 1982). The optimal conditions for the embryos to develop and survive are between 20 to 30 ppt salinity and between 20 to 30°C temperature (Harris, 1982). At 28°C and 30 ppt, the development of the embryos takes between 14 and 17 days; it takes longer at lower temperatures (Kennedy et al., 2007). Considering that courtship and mating in the blue crab primarily take place in the oligo- and mesohaline regions of estuaries, where salinities often fall below tolerance limits for larvae and early juveniles, newly inseminated females must migrate from mating sites in the upper and middle estuary to locations near the estuary mouth and the adjacent ocean. This migration, enabled by their well-developed osmoregulation system and broad

temperature tolerance, ensures optimal conditions for embryo hatching and larval release (Epifanio, 2019).

Before migration, which occurs in two distinct phases, the female undergoes a recovery period post-molt, during which she accumulates the necessary energy reserves. In large estuaries, this migration can span over 200 km (Epifanio, 2019). Migration begins after mating in early autumn as water temperatures drop and daylight hours shorten. This process is well-documented in the Chesapeake Bay, where, in the first phase, females move along the edges of the deep channel that defines the main stem of most estuaries (Epifanio, 2019; Kennedy et al., 2007). During this phase, many females overwinter; when water temperatures fall below 10°C, they cease movement and burrow into the sediment to spend the winter, re-emerging in the spring as temperatures rise (Epifanio, 2019; Kennedy et al., 2007). At this time, females prefer sandy sediments mixed with silt, which initially facilitates winter burial and later aids in the adhesion of fertilized eggs to the pleopods (Kennedy et al., 2007).

After overwintering, females resume their migration, reaching areas near the estuary mouth by early to mid-summer, where the salinity is suitable for the final maturation and fertilization of the eggs (Epifanio, 2019). The fertilized eggs are transferred to the ventral surface of the abdomen via gonopores, attaching to the hairs of the pleopods (Epifanio, 2019; Kennedy et al., 2007).

The female carries the embryos under her abdomen throughout embryonic development, which lasts between two and three weeks (Epifanio, 2019).

In the second phase of migration, ovigerous females move from the lower estuary to the adjacent coastal ocean, to hatch their eggs and release larvae in the water column. During this phase, ovigerous females exhibit specific behaviors in response to tidal and lunar rhythms, as well as photoperiod (Epifanio, 2019; Kennedy et al., 2007). During this second phase of migration, the females exhibit a characteristic movement pattern. Specifically, they utilize one tidal phase for movement, while remaining on the bottom during the other. Several studies have concluded that ebb-tide transport is used by ovigerous females to reach the coastal ocean, while females that have already released their larvae use flood-tide transport to return to the estuary (Epifanio, 2019).

1.3.3 Gonads

- **Female**

The ovaries and the seminal receptacles are the two paired structure that characterized the female internal reproductive system. Together, the two gonads form an H-shape (Kennedy et al., 2007). Prior to the pubertal molt and during copulation, the ovaries are immature, containing only minute, undeveloped eggs and appearing as small pink filaments (Brown, 2009; Kennedy et al., 2007). In the months following copulation, oogenesis occurs in

the gonads, a sequence of stages that, from oogonium, lead to the mature oocyte (Kennedy et al., 2007). During this process, vitellogenesis takes place, forming yolk, which causes the ovary to increase in size and develop an orange color. There are two types of cells in the developing ovary: oocytes, in different development stages, and accessory cells, also named nurse cells or follicle cells (Brown, 2009; Kennedy et al., 2007).

In blue crabs, the ovary is surrounded by a thin, visible layer of connective tissue that separates it from the hemocoel. The ovary is enclosed within structures called ovarian lobes or ovarian pouches, which contain the oocytes (Brown, 2009; Kennedy et al., 2007).

The majority of oocytes in the ovarian lobes are at roughly the same developmental stage. Oogenesis is a continuous and relatively rapid process, where oocytes progress from primary growth to secondary growth (Brown, 2009). While some lobes primarily exhibit two main stages, they may also contain a small percentage of oocytes at a third stage (Brown, 2009). The development of oocytes is fluid, with oogonia always present, though not always noticeable. As oogenesis progresses, oocyte development occurs from the center of the ovarian lobe outward (Brown, 2009). Thus, in a mature ovarian lobe, the mature oocytes are positioned at the periphery, while the center contains a row of germ cells, namely oogonia and early primary growth

oocytes. This central area, known as the germinal zone, is present in all ovarian lobes and supply new cells for the ongoing development of oocytes (Brown, 2009). During primary growth, germinal zones are poorly defined, making oogonia hard to identify. In secondary growth, however, the germinal zones primarily consist of primary growth oocytes and become well-defined (Brown, 2009).

The seminal receptacles are located at both distal ends of the oviduct within a hard, chitinous wall. Their size and color vary based on the maturity of the ovary (Brown, 2009). Initially, they appear as small, thin, white, and membranous disks, gradually transforming into large, hard, pink rounded spheres. The spermathecae store sperm and seminal fluid, serving as the fertilization site in the blue crab, where sperm can survive for at least a year (Brown, 2009; Millikin, 1984).

In Chesapeake Bay, while some female blue crabs can copulate and spawn in the same season, the majority undergo overwintering after copulation, delaying spawning until the following spring and summer (Kennedy et al., 2007). Throughout the winter and early spring, the seminal receptacles gradually shrink, while the ovary continues to thicken and elongate (Brown, 2009; Kennedy et al., 2007). Research conducted in Florida indicates that females can produce as many as eight consecutive broods over a period of 4 to 6 months,

with intervals as short as 10 to 14 days between the release of larvae from one brood and the commencement of another (Kennedy et al., 2007).

- **Male**

The male reproductive system of *Callinectes sapidus* is located in the dorsal part of the thoracic cavity, closely associated with the hepatopancreas, similar to the female system (Carvalho-Saucedo et al., 2015). The internal structures form a bilateral 'H' shape and consist of paired testes, vasa efferentia, and vasa deferentia. The vasa deferentia are divided into three main sections: anterior, middle, and posterior (Kennedy et al., 2007).

The testes are tubular structures consisting of testicular lobes that surround a central seminiferous duct. Within these lobes, spermiogenesis occurs, encompassing the development of spermatogonia into spermatocytes, spermatids, and finally mature sperm, which are typically found at the center of the lobes (Kennedy et al., 2007). As the testicle matures, it develops an intense pink hue (Carvalho-Saucedo et al., 2015). The seminiferous duct is enclosed by an epithelium except where it opens broadly into the testicular lobes, facilitating the passage of mature sperm into the duct. The seminiferous duct contains only mature sperm (Kennedy et al., 2007).

From the testes, mature sperm pass through the anterior vas deferens (AVD), highly coiled tubules that encapsulate them into ovoid-shaped structures called spermatophores and then transport them to the middle vas deferens (MVD) (Kennedy et al., 2007). The AVD is the primary storage area for fully formed spermatophores (Millikin, 1984). The largest parts of the male reproductive system are the MVD, which feature numerous large coils and many lateral out-pockets. The seminal fluid, a granular and pink secretion, fill the large lumen of each coil. The posterior vas deferens (PVD), linked with the ejaculatory duct, then conveys the ejaculate to the external penes (Kennedy et al., 2007).

1.4 Environmental factors influencing the reproduction

For ectothermic species like *Callinectes sapidus*, temperature significantly affects various physiological processes such as growth, development, reproduction, and likely egg production (Darnell, 2009). Since sexual maturity is closely associated with body size, factors like temperature, salinity, and the quality and quantity of food, that influence growth and molting, also determine the timing of sexual maturity (Kennedy et al., 2007). In males, environmental conditions play a crucial role in the onset of reproductive readiness, resulting in seasonal variations in the condition of both internal and external reproductive systems (Kennedy et al., 2007). In late autumn and winter, the internal reproductive system becomes inactive, as indicated by the cessation of sperm

production in the testis and the absence of seminal fluid in the middle vasa deferentia. In contrast, males of the tropical portunid species *Callinectes ornatus* exhibit a continuous cycle of gonad maturation year-round (Kennedy et al., 2007). In females, sexual maturity is established at the terminal molt; the sizes of mature female blue crabs, as well as those of other crab species, exhibit significant seasonal and spatial variation both among and within estuaries. This pattern strongly suggests that environmental factors have a substantial impact on the timing of sexual maturity (Kennedy et al., 2007).

It has been observed that the spawning season significantly shortens during years with prolonged low temperatures. Mating mainly takes place in the upper areas of estuaries and the lower portions of rivers, where the water is relatively low in salinity (Hill, 1989). By influencing molting behavior, which determines sexual maturity and the timing of mating, environmental factors (primarily temperature and salinity) can affect the mating of blue crabs. In temperate regions, blue crabs typically mate during the warm summer months, whereas in subtropical areas, mating can begin much earlier (Kennedy et al., 2007).

Molting does not occur below approximately 9°C, resulting in temporal variations in the distribution and abundance of pre-pubertal females. For example, in late spring, the warmer waters of lower Chesapeake Bay see a high number of pre-pubertal females prepared for their pubertal molt. Conversely,

in the colder waters further up the bay, there are significantly fewer receptive females (Kennedy et al., 2007).

Consequently, there are two distinct periods of mating activity in the lower bay: one in the spring and another from late summer to early fall. In contrast, the mid and upper bay experience a single peak in mating activity from mid to late summer (Kennedy et al., 2007). For example, in the spring, a higher number of females may remain unmated or receive less sperm and seminal fluid because there are fewer large males or recently unmated males available compared to the late summer (Kennedy et al., 2007).

Salinity also plays a crucial role in the spatial and temporal distribution of sexually receptive crabs, thereby dictating mating opportunities. In a sub-estuary of Chesapeake Bay, the Rhode River, low salinity areas are primarily utilized by pre-pubertal males for molting during the summer. In contrast, brackish areas are favored for mating and are predominantly occupied by intermolt males and pre-pubertal females (Kennedy et al., 2007).

Given that many physiological processes are influenced by environmental factors, using days as a measure for assessing clutch timing is not physiologically accurate because these processes slow down at lower temperatures. While total egg production is strongly correlated with lifespan, both clutch size and the timing of egg production are affected by water

temperature (Darnell, 2009). Female blue crabs can store sperm from a single mating event that occurs after their terminal molt. This sperm can remain viable for several months to over a year. However, during this storage period, sperm cells are exposed to oxidative stress, osmotic changes, temperature fluctuations, and ATP depletion, which can diminish their viability (Darnell, 2009).

Crabs that matured between July and November were significantly more likely to survive into a second season compared to those maturing earlier in the year. This is because crabs maturing later experience fewer warm days between their terminal molt and the onset of winter (Darnell, 2009). Cooler water temperatures in the fall and winter tend to slow down both egg production and metabolism, delaying these processes until spring (Darnell, 2009).

It has been found that blue crab eggs hatch only within a specific range of salinities (23-33 ppt) and temperatures (19-29°C). Additionally, egg mortality can occur due to fungal infections, predation, suffocation in stagnant water, and exposure to extreme temperatures. On average, only one in every million eggs survives to reach maturity (Hill, 1989).

1.5 Blue crab as an alien species

Invasive alien species (IAS) are non-indigenous species that have negative effects on ecosystem functioning, biological diversity, human health, and/or

socio-economic values in invaded regions. The invasion by these species over the past decade has become a central environmental issue (Mancinelli et al., 2013). Areas particularly affected by biological invasion are the Mediterranean Sea and southern European waters (SEW), where both the rate of introduction and the number of these species are of concern (Mancinelli et al., 2013). In fact, IAS have been recognized as a biological pressure by several European reference regulations such as the Marine Strategy Framework Directive (MSFD; 2008/56/EC), particularly in descriptor 2 (D2) and the Biodiversity Strategy to 2020 [2011/2307(INI)] (Mancinelli et al., 2013).

Crustaceans exemplify this ongoing invasion process. The Atlantic blue crab, *C. sapidus*, is found in seven of the nine South-European Marine Ecoregions (Mancinelli et al., 2017b). It is listed among the 100 worst invasive alien marine species in the Mediterranean due to its harmful impacts on human activities. The crab's ability to attack other crustaceans and fish trapped in fishing nets and its tendency to damage fishing gear contribute to its significant social and economic impact, in addition to its adverse effects on native biodiversity (Piras et al. 2019).

To address the spread of the blue crab, an invasive species, the Italian government has enacted a law providing financial support for businesses involved in capturing and disposing of the blue crab. As stated in the Ministerial

Decree implementing Article 10 of Decree-Law No. 104 of August 10, 2023, on 'Countering the Spread of the Blue Crab *Callinectes sapidus* and *Portunus segnis*,' a total of 2.9 million euros is allocated to cover up to 80% of the costs for capture and 100% of the costs for disposal. Additionally, the aquaculture sector, which has also been severely affected by this invasive species, will receive support. To aid the impacted aquaculture businesses, a 50% discount on social security contributions for December 2023 has been introduced. This discount applies to companies classified under ATECO code 03.21. A fund of 500,000 euros has been allocated to finance this assistance (Gazzetta Ufficiale della Repubblica Italiana, Serie Generale, n. 237 del 15 dicembre 2023).

C. sapidus is native to western Atlantic basin; within this range, it serves a crucial function in benthic ecosystems both as a keystone consumer and as a prey for various fish. This makes the blue crab a vital connector between benthic and pelagic food webs, integrating different parts of the marine ecosystem (Türeli et al., 2016; Scalici et al., 2022).

In non-native range, invasive species can drastically transform the ecosystems, exhibiting behaviors and dynamics different from those in their native area due to the absence of competitors or predators. These species are usually present in estuarine and marine ecosystem, where they reduce the abundance of native species, leading to changes in the food web (Türeli et al., 2016).

Over the past century, the blue crab has increasingly appeared in Africa, Asia, and Europe. The pathways of its introduction are unclear; it is likely that its long larval stage and tolerance to various salinities suggest it could spread through marine currents or ship ballast water. Another hypothesis is that its value as seafood may have led to intentional releases to establish breeding populations in new areas (Schubart et al., 2022).

The colonization of European waters began in 1900 along France's Atlantic coast, later spreading to the Baltic Sea, the North Sea, and down to Portugal's Atlantic coast (Schubart et al., 2022). In the Mediterranean Sea, it was first recorded in 1948 in the northern Adriatic Sea, with earlier suspicions of its presence in the Aegean Sea in 1935 (Piras et al., 2019). Additionally, it has spread to Japan and the Indo-Pacific Ocean, as well as northward in its native range, likely driven by sea water warming (Mancinelli et al., 2021; Schubart et al., 2022).

The success of non-indigenous species (NIS) like the blue crab depends on the ecological characteristics of the new habitat and the species' ecological plasticity, including reproductive capacity, behavior, trophic flexibility, and response to environmental stress (Scalici et al., 2022). Its omnivorous diet, high mobility, large body size, high fecundity, and aggressive behavior enhance its potential for successful colonization (Schubart et al., 2022).

In the past decade, numerous studies have highlighted the high nutritional value of Mediterranean blue crab meat, leading to the establishment of small fisheries, particularly in northern Greece and Turkey, where it has been documented in 15 lagoon systems (Türeli et al., 2016; Piras et al., 2019).

CHAPTER II

AIM

The aim of this thesis is to explore the reproductive strategy of the blue crab (*Callinectes sapidus*) in the Adriatic Sea. Native to the Western Atlantic Coast, this species is now recognized as one of the 100 worst invasive alien marine species in the Mediterranean Sea. Its rapid proliferation has led to significant disruptions not only to local human activities but also to marine ecosystems. The blue crab's aggressive predation on other crustaceans and fish, combined with its tendency to damage fishing gear, has caused considerable economic and social impacts. Equally concerning are the severe alterations it induces in the structure and composition of coastal marine and freshwater communities, leading to adverse effects on native biodiversity.

Despite its considerable impact, the reproductive behavior of the blue crab in the Adriatic has not been studied. This thesis aims to address this knowledge gap by comparing the reproductive strategies of the blue crab in the Adriatic with those documented in its native Atlantic range. By employing histological analysis of gonads, this thesis seeks to provide insights into the reproductive mechanisms of the blue crab in this non-native environment. Understanding these reproductive patterns is crucial for developing effective management

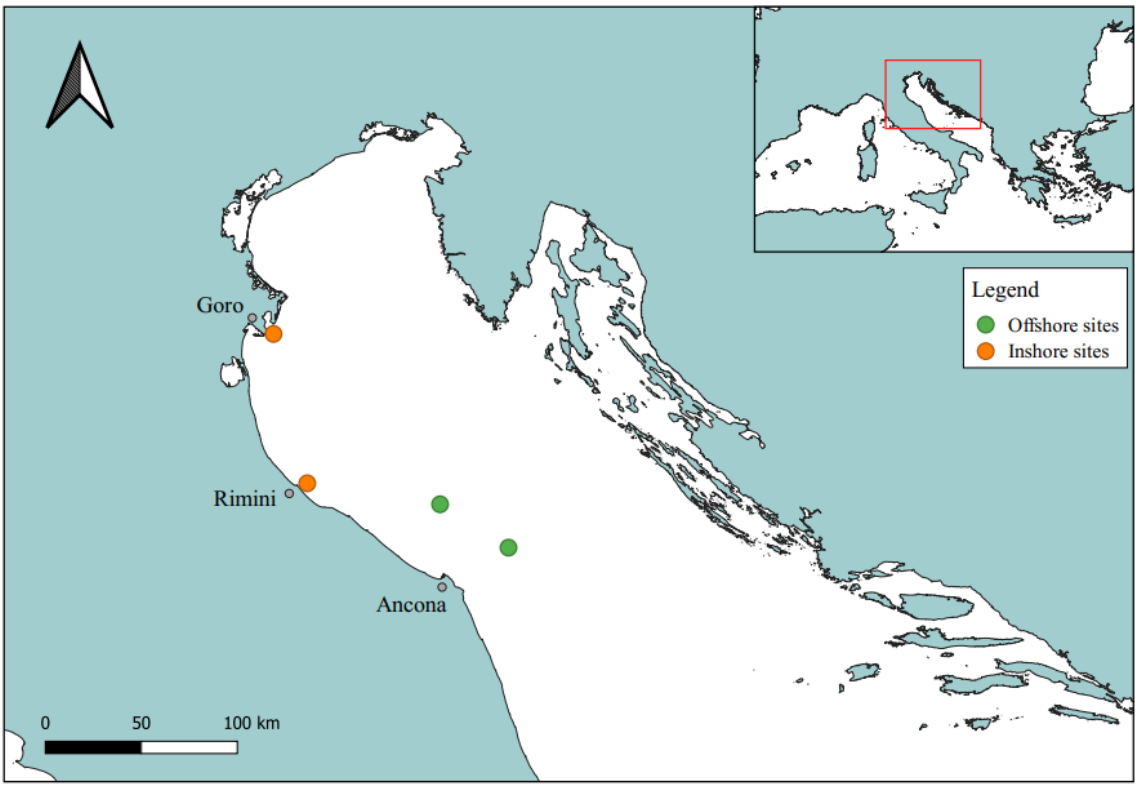
strategies to control its spread and mitigate its negative effects on both the region's marine ecosystems and local fisheries.

CHAPTER III

MATERIALS AND METHODS

3.1 Sample collection sites

The samples of *Callinectes sapidus* were collected by local fishermen between September and November 2023 in the Adriatic Sea (FAO-Geographical Sub-Area 17, according to GFCM division). The individuals were sourced from four different sites, categorized as offshore and inshore. Specifically, they were caught by two different vessels (Destriero and Primula Rossa) offshore near Ancona (AN), while inshore samples were obtained from the Goro Lagoon (FE) and the coast of Rimini (RN). The offshore samples were collected using trawling methods. The samples, once caught, were kept at 4°C on the boat and maintained this temperature until their arrival at the laboratory. The uninterrupted cold chain ensured that the samples remained in good condition for subsequent analysis.



3.2 Sampling

During the sampling, various data were collected, including sex (determined by the shape of the abdomen), total weight, weight of embryos, weight of gonads, carapace length, and carapace width (**Fig. 15**).



Figure 15. Equipment used for sampling and collecting biometric data included scales, calipers, tweezers, rulers, and scissors.

The individuals were dissected to obtain gonad samples, which were weighted and then fixed in formalin, inside a Falcon tube (15mL) and stored at 4°C for subsequent histological analysis.

3.3 Histological analysis

3.3.1 Paraffin inclusion

The first phase of the protocol involves the dehydration of the samples, which are placed in biocassettes and then dehydrated through a series of baths in ascending concentrations of ethanol (70%, 80%, 95%, 100% I and 100% II). Subsequently, the protocol requires two passes through xylene (BioOptica, Milan, Italy) baths. The final step involves embedding the sample in paraffin (BioOptica).

3.3.2 Microtome

Once the sample is fixed and embedded into paraffin, solidified paraffin blocks were cut into 5 μm sections using a microtome (Leica RM2125 RTS, Nussloch, Germany). The slices are immersed in a deionized water bath before being transferred to clean slides. The slides with the paraffin sections are then placed in a warm environment to allow the tissue to adhere to the glass.

Six slides were prepared for each specimen, with three sections on each slide.

3.3.3 Staining technique

The sections were then stained according to two different staining protocols: 3 slides were prepared for each staining method.

The staining methods used are:

- Hematoxylin and eosin stain (Merck KGaA): hematoxylin colors in blue violet the basophilic cellular components, such as nucleic acid in the cell nucleus, while eosin colors in pinkish red acidophilic components, like the cytoplasm and extracellular matrix.
- Periodic Acid Schiff (PAS, Bio-Optica): is used to detect polysaccharides, muco-substances, and glycogen in tissues. It stains these substances magenta, highlighting structures such as basement membranes and glycogen deposits.

In both staining protocols, there are two steps where the slides with sections are placed in two consecutive Xylene baths to allow for deparaffinization of the sections. Then, the samples undergo a decreasing series of alcohol baths (100% I, 100% II, 95%, 80%, and 70%), and after staining, an increasing series of alcohol baths (70%, 80%, 95%, 100% I, and 100% II). After the series of ethanol baths, the sections are placed again in xylene before mounting; during this final phase, a glue is used to secure the cover slips to the slides.

Finally, the sections were examined with an optical microscope (Zeiss Axio Imager.A2, Oberkichen, Germany), and images were captured with an Axiocam 503 digital color (Zeiss, Oberkochen, Germany).

CHAPTER IV

RESULTS

During the three-month sampling period, at the conclusion of the spawning season, a total of 248 individuals were collected, comprising 217 females and 31 males. A greater number of females were found offshore compared to inshore, whereas the opposite trend was observed for males; indeed, very few males were captured offshore (**Fig. 16**). This is corroborated by the sex ratio distribution, which reveals an equal distribution of 50% males and 50% females inshore, in stark contrast to the significant predominance of females offshore.

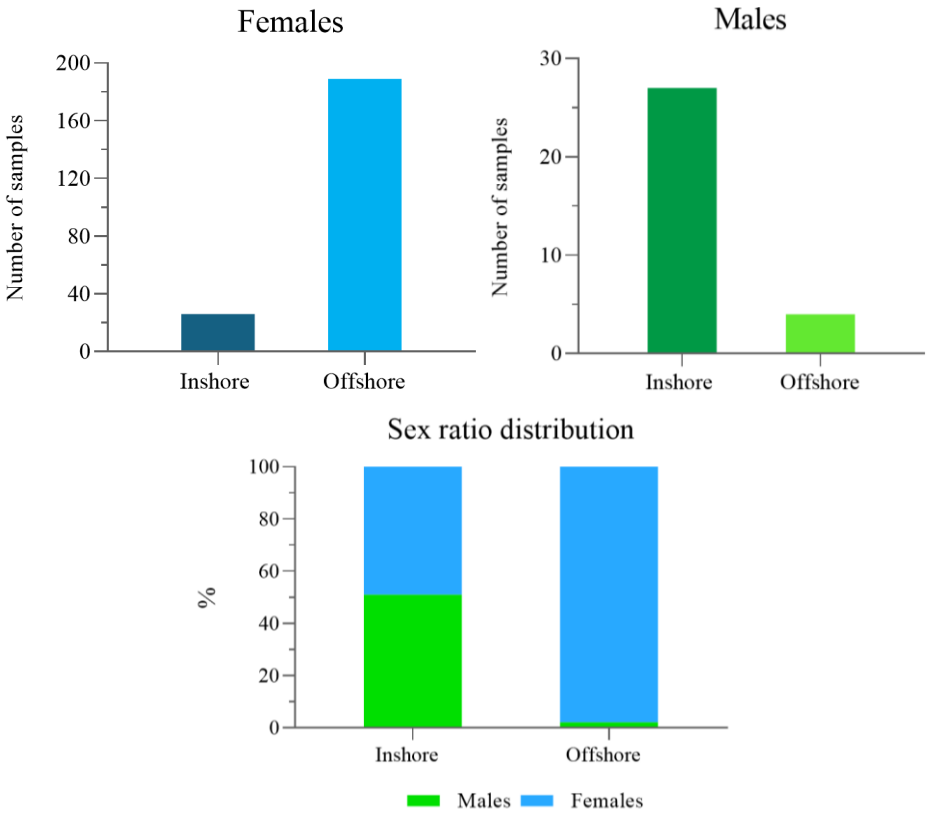


Figure 16. Number of females and males and sex ratio distribution of *C. sapidus* caught inshore and offshore.

The individuals, both female and male, were classified into three distinct size classes: <100g, 100g-150g, and >150g (**Fig. 17**). Examining the frequency of individuals within these size classes reveals no significant differences among females. The highest number of females found inshore was in the <100g category, whereas offshore, the majority was in the 100g-150g category. Conversely, notable differences in size were observed among males. Offshore, only males >150g were present, while inshore, males were found across all three size categories.

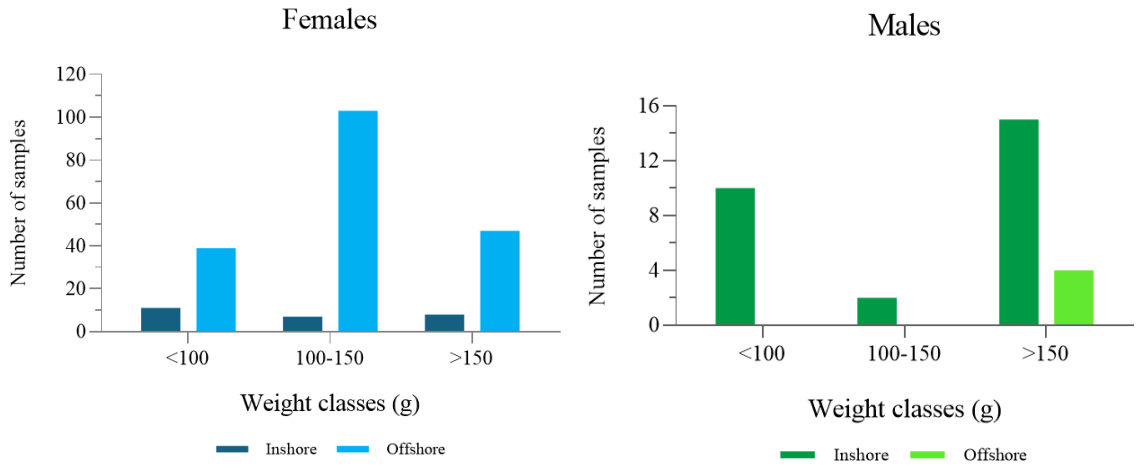


Figure 17. Females and males of *C. sapidus* distinct in three size classes (<100g; 100g-150g; >150g) and divided in inshore and offshore.

Subsequently, the biometrics of both females and males, distinguished between inshore and offshore, were analyzed in detail (**Fig. 18**). The average total weight, average gonad weight, average carapace width, and average carapace

length were evaluated. No significant differences were observed in either females or males.

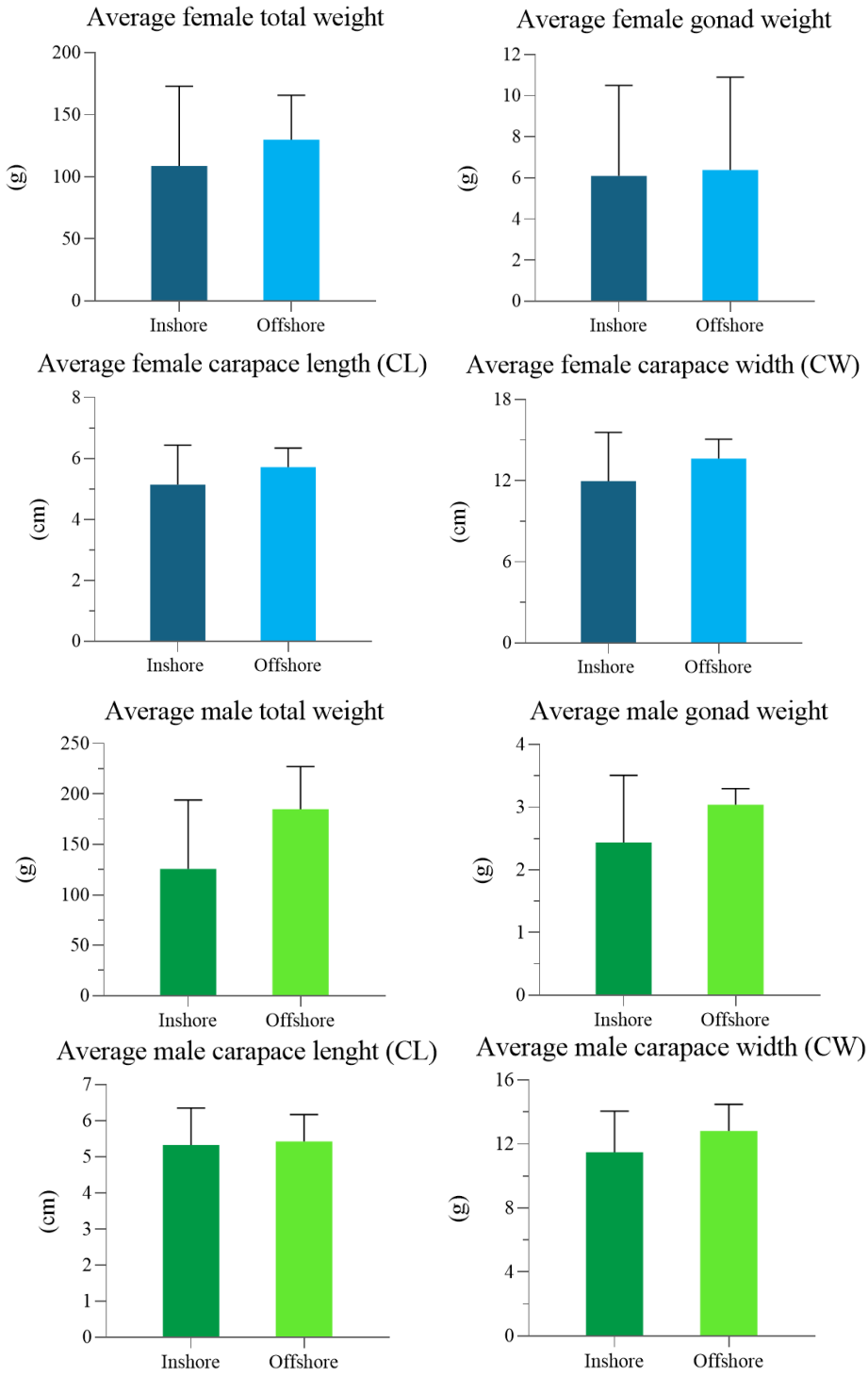


Figure 18. Females and males *C. sapidus* biometrics: average total weight; average gonad weight; average carapace width (CW) and average carapace length (CL).

In analyzing biometric data across different weight classes for both sexes, significant differences were observed only for the carapace width (CW) and carapace length (CL) of females in the <100g weight class, and for the carapace length (CL) of males in the >150g weight class (**Fig. 19**).

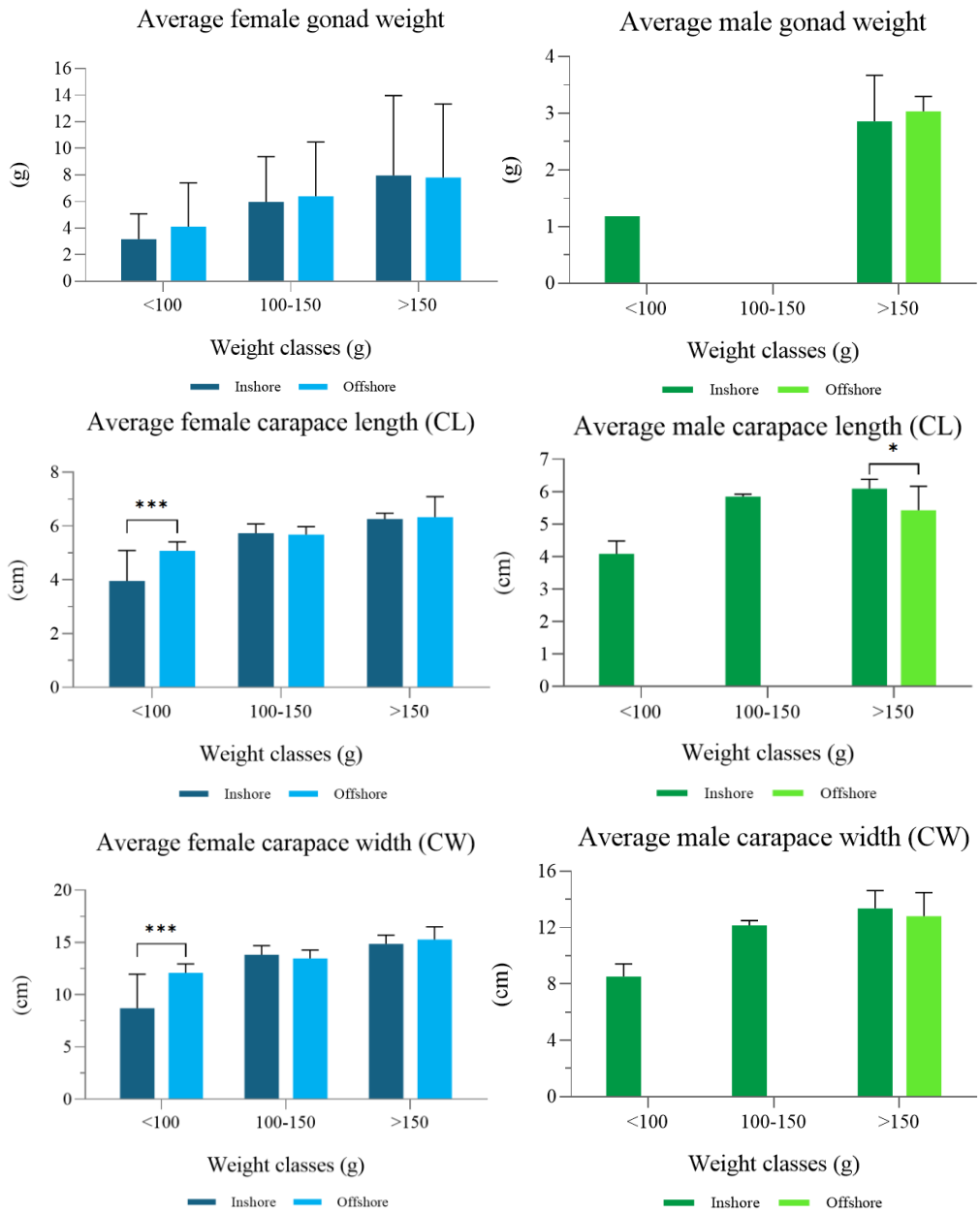
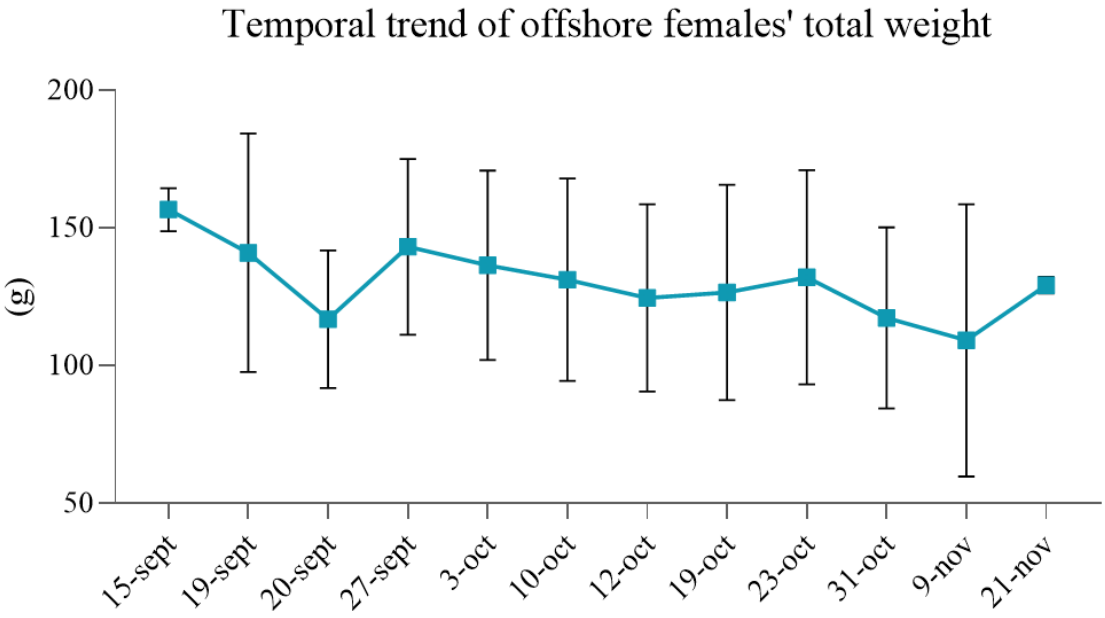
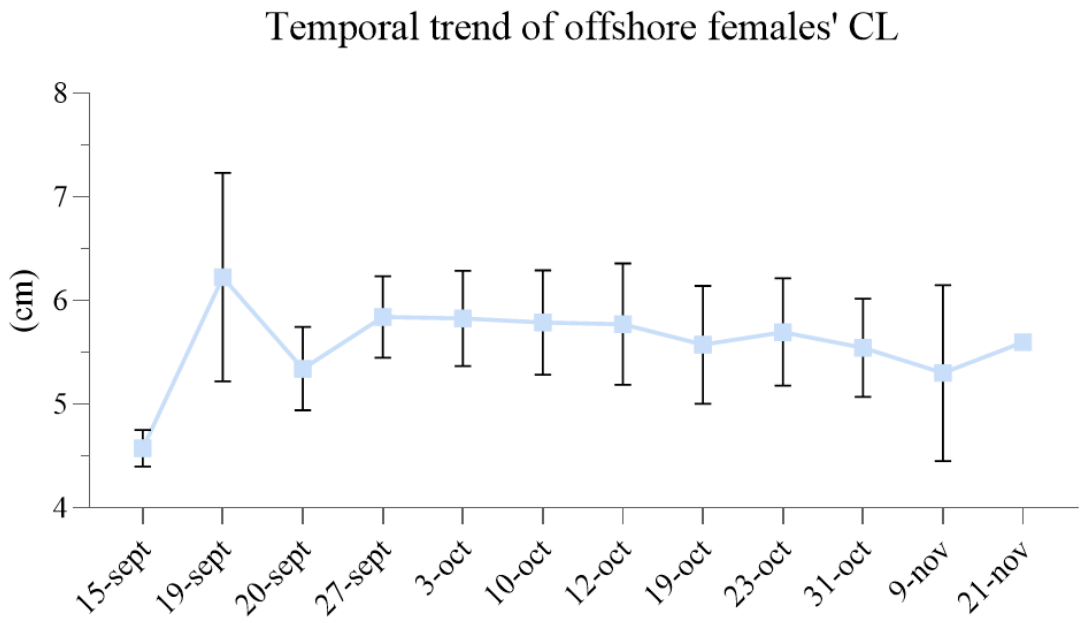


Figure 19. Females and males *C. sapidus* biometrics (average total weight; average gonad weight; average carapace width and average carapace length) divided in size classes.

By analyzing the data for each individual sampling, it was possible to establish a temporal trend (**Fig. 20**). This analysis was conducted for offshore females, focusing on total weight, carapace width, and carapace length, to observe how these values vary over the sampling period.





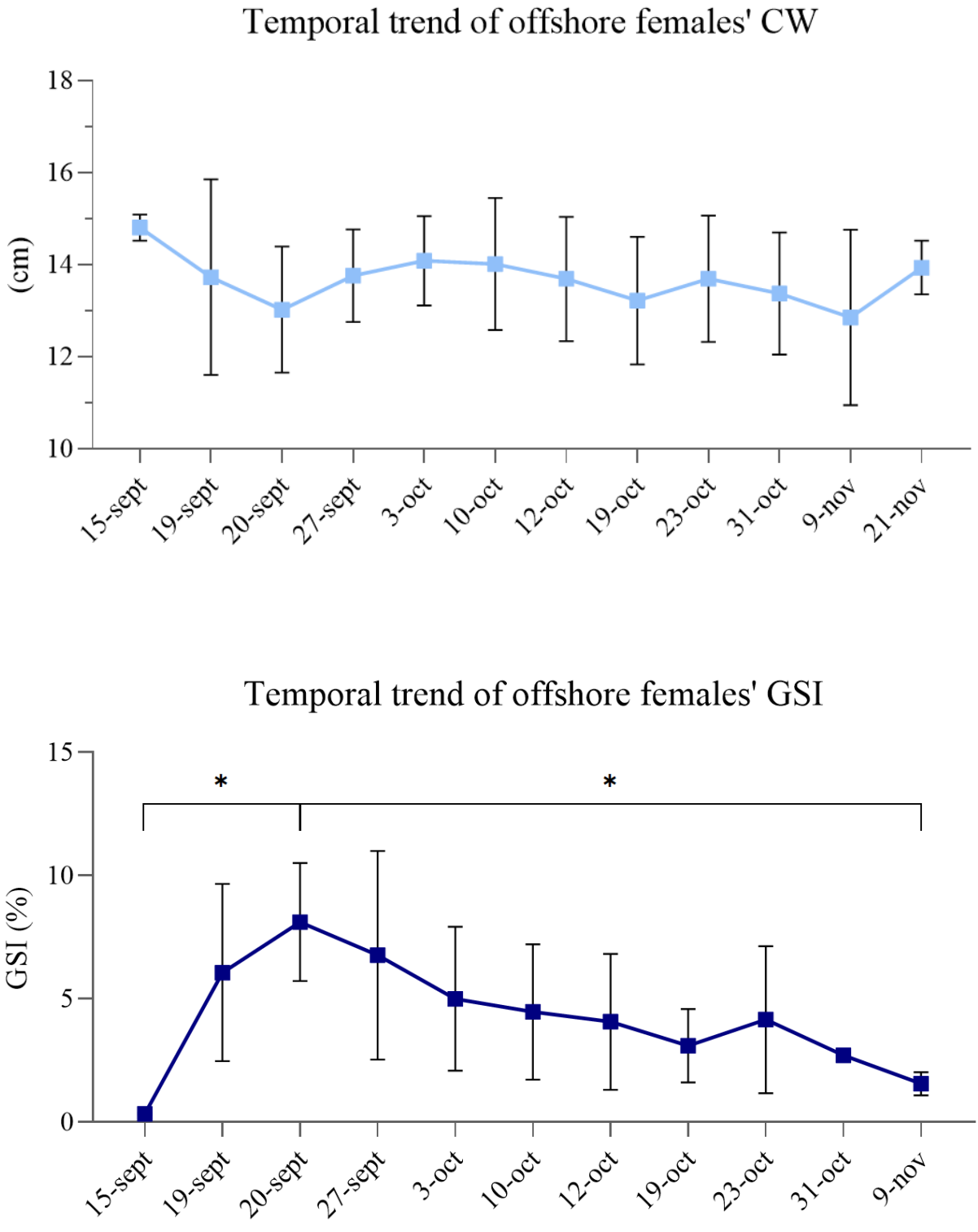


Figure 20. Temporal trend of offshore females' total weight, CL (carapace length), CW (carapace width) and GSI. The values used for temporal trends represent the average values for each sampling day.

The Gonad Somatic Index (GSI) was subsequently evaluated, calculated as the ratio of gonad weight to total weight, multiplied by 100 (**Fig. 21**). It was observed that offshore females had higher GSI values compared to inshore females, whereas the opposite was observed in males. Further analysis of GSI across different size classes revealed that offshore females exhibited higher GSI values in the <100g and 100g-150g size classes, while inshore females had higher GSI values in the >150g size class. For males, a higher GSI value was noted in the <100g size class; however, within this class, only inshore samples were available for evaluation.

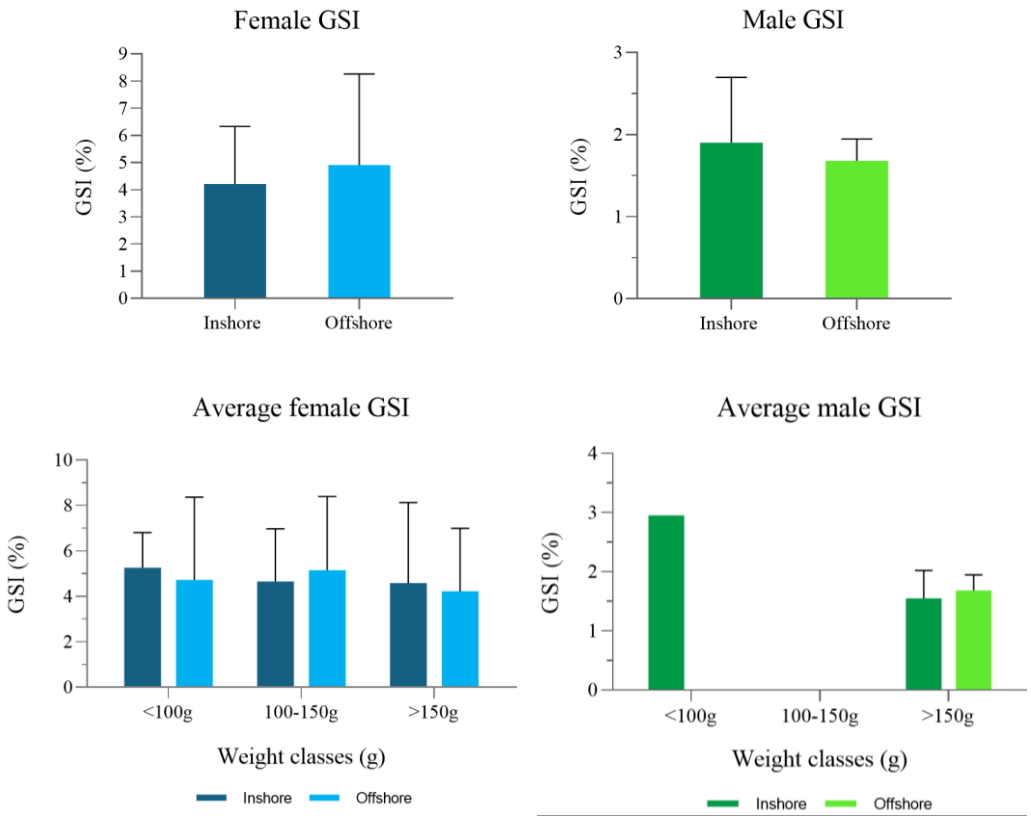


Figure 21. Average GSI of *C. sapidus* females and males, separated in inshore and offshore and by size classes.

Finally, the developmental stage of the gonads was assessed in a representative subsample of female (**Fig. 22**). No inshore females were found to have embryos. Upon characterizing the gonadal stages, it was observed that 40% of inshore females were immature, 40% were in a post-spawning condition, and 20% were in late secondary growth. In contrast, offshore females were found both with and without embryos. Specifically, females in late primary growth did not have embryos, whereas those in early secondary growth and mid secondary growth all contained embryos. In late secondary growth, full growth, and post-spawning stages, both types of samples - those with and without embryos - were present.

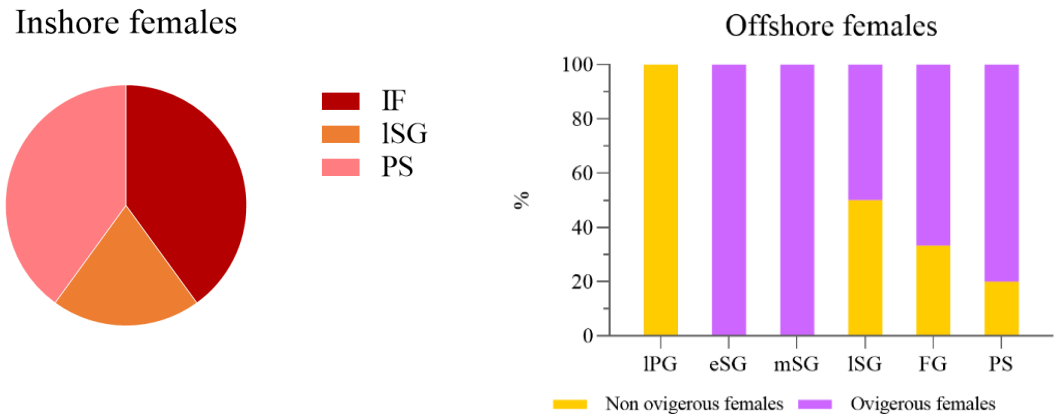


Figure 22. Developmental stages of the female gonad in blue crab individuals are depicted. The upper portion shows inshore females, whereas the lower portion illustrates offshore females, differentiated into ovigerous and non-ovigerous (with and without embryos). **IF**= immature females; **lPG**= late primary growth; **eSG**= early secondary growth; **mSG**= mid secondary growth; **lSG**= late secondary growth; **FG**= full growth; **PS**= post spawning.

Among offshore post-spawning females, three distinct conditions can be observed. In one case, there are females without eggs and unable to produce

additional embryos due to the absence of a germinative zone (**Fig. 23A**). Another condition involves females with embryos but with low reproductive potential (**Fig. 23B**). Finally, there are females with eggs that can undergo another spawning event (**Fig. 23C**).

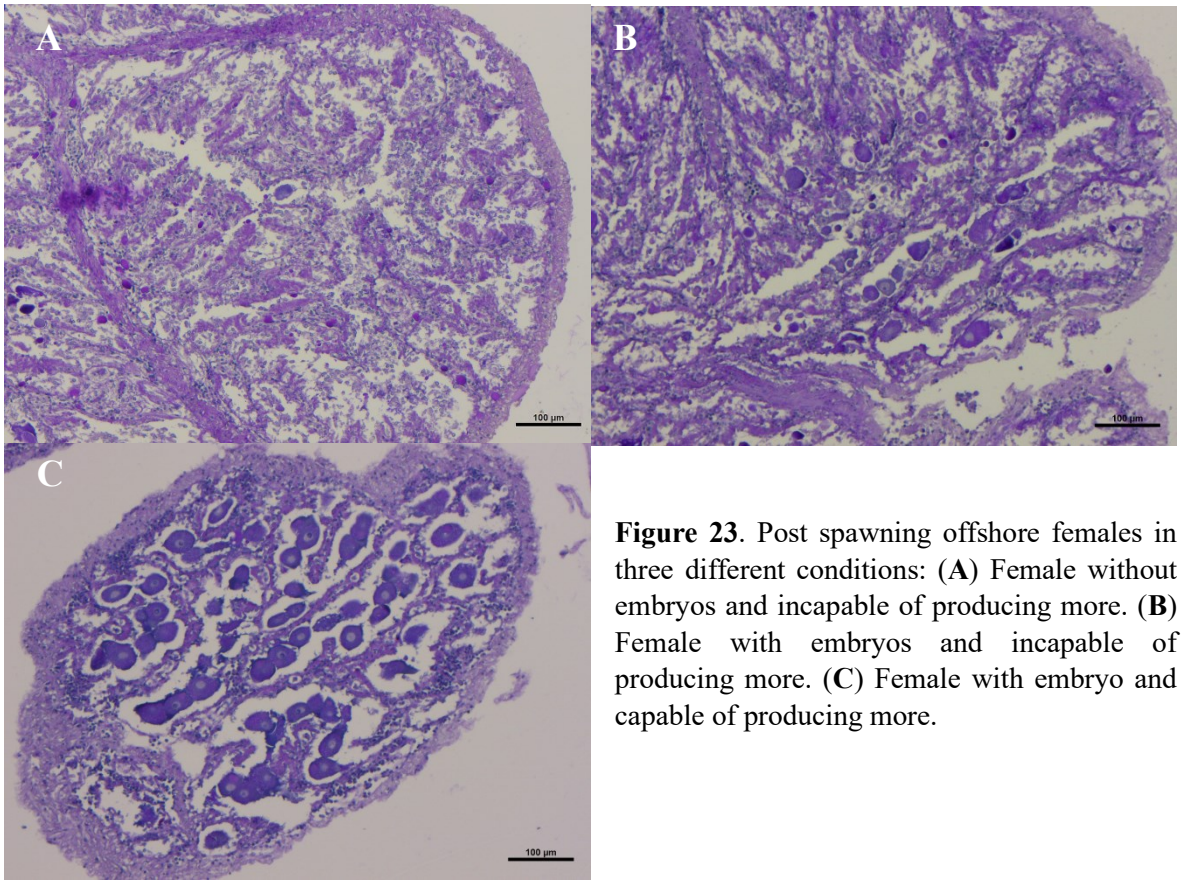
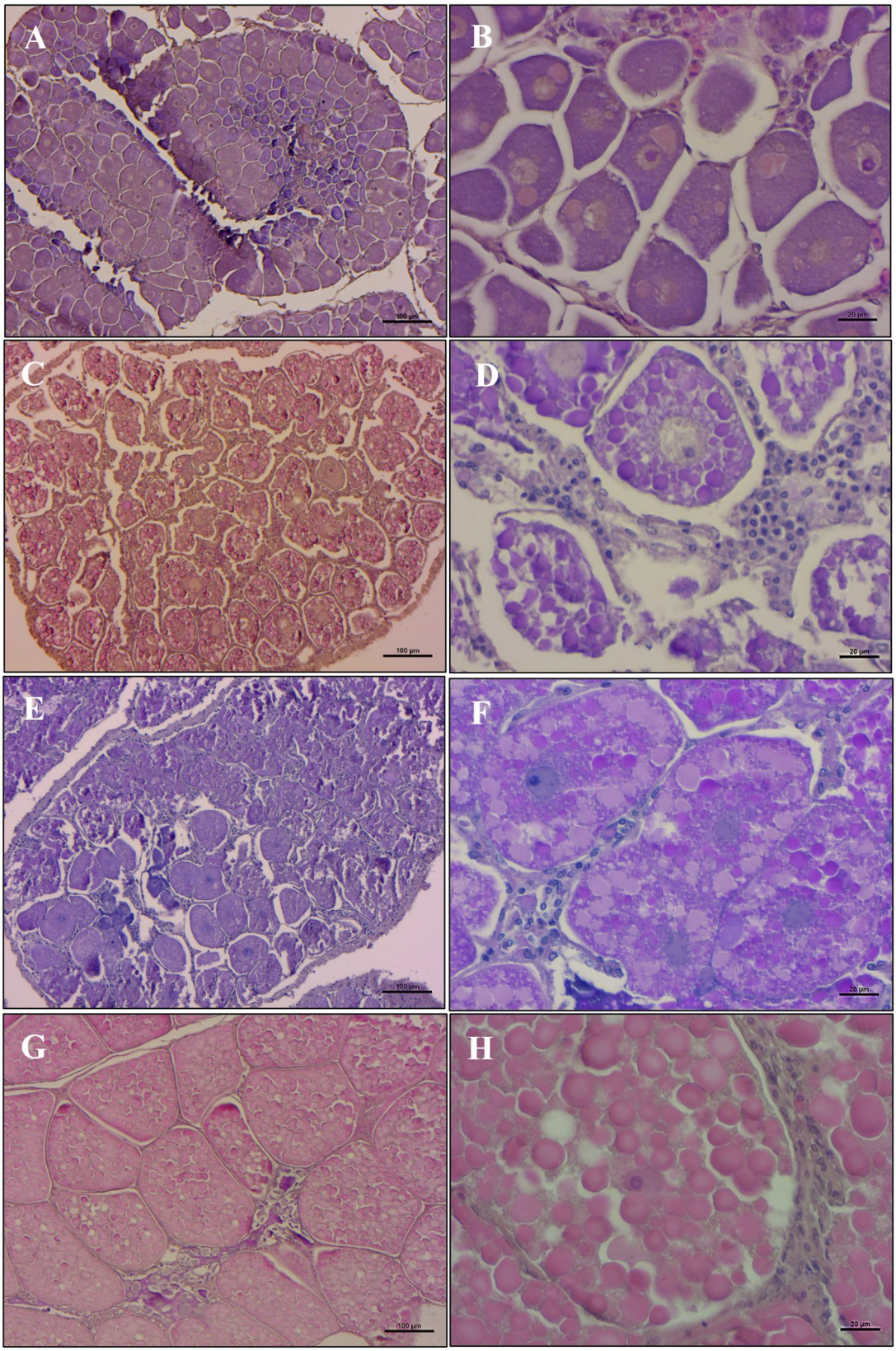


Figure 23. Post spawning offshore females in three different conditions: (**A**) Female without embryos and incapable of producing more. (**B**) Female with embryos and incapable of producing more. (**C**) Female with embryo and capable of producing more.

Representative images of the different developmental stages of the female gonad in blue crabs observed in the analyzed samples are provided (**Fig. 24**).


A
B
C
D
E
F
G
H

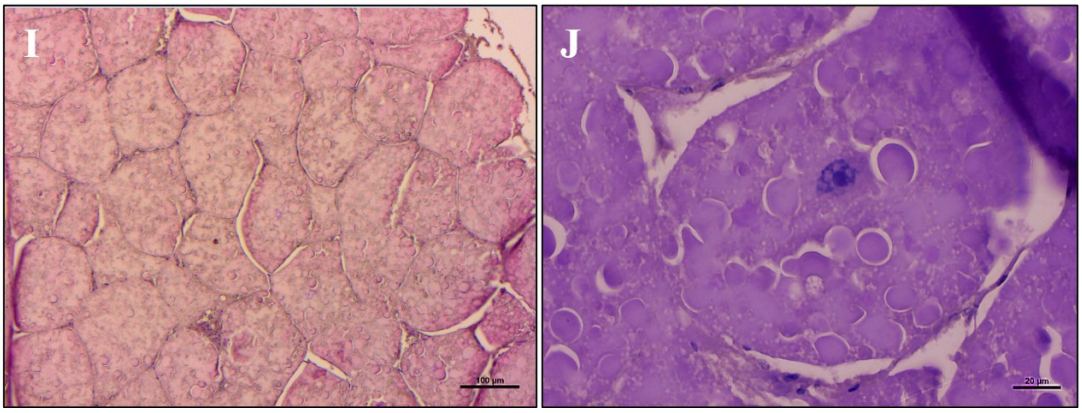


Figure 24. A, B = late primary growth (lPG) basophilic ooplasm that stains blue, the perinuclear yolk complex (PYC) is present, oil droplets and cortical alveoli begin to form, the germinal zone (GZ) is not very evident; **C, D = early secondary growth (eSG)** acidophilic ooplasm that stains pink, presence of yolk globules, the GZ is well evident, as are the oil droplets; **E, F = mid secondary growth (mSG)** acidophilic ooplasm that stains pink, presence of yolk globules, the GZ is well evident, but the oil droplets are no longer evident; **G, H = late secondary growth (lSG)** the yolk globules increase in number and size, the oocytes are full of yolk, and the GZ is less evident; **I, J = full growth (FG)** ooplasm mostly light pink, the yolk globules fuse starting from the periphery and then towards the center.

CHAPTER V

DISCUSSION

The blue crab has been present in the Mediterranean Sea for a long time, and there is significant concern about this species, particularly due to the damage it causes to human economic activities and to ecosystems. Despite the growing attention over the years, there remains a lack of clear understanding regarding the reproductive period of this species and its reproductive strategy in the Mediterranean Sea, especially in the Adriatic Sea.

The sampling period for this study was limited to just three months, coinciding with the latter part of the blue crab's reproductive season. The sampling period included the months of September, October, and November 2023. It was not possible to collect data in August because, in the northern central Adriatic Sea, from San Benedetto del Tronto (AP) upwards, there is a fishing ban that typically lasts until mid-September. As a result, it was impossible to collect samples of blue crab during this period. In November, however, only a few samples were collected, suggesting that the area of interest for the fishermen from whom the samples were taken was no longer inhabited by spawning females. The absence of spawning females indicates either that they have migrated to another spawning area or that the reproductive season has ended (Darnell et al., 2009). Macroscopic observation suggests that the reproductive

season ends there. Indeed, the females caught in November had small and transparent gonads, even in the samples without eggs, indicating they were not ready for another spawning event. Based on the results obtained, we can hypothesize that the reproductive season in that area ends in mid-November. Nevertheless, we do not know if this is the only spawning area during the previous months.

The reproductive period of the blue crab varies significantly depending on the region, influenced by environmental conditions and exhibiting both temporal and spatial variations in the timing and duration of the spawning season. Generally, brood production begins earlier in the spring at lower latitudes (Kevrekidis et al., 2023). For instance, in the Monolimni Lagoon (North Aegean Sea), the reproductive period has been observed to extend from May to October, with a peak in September, coinciding with the onset of autumn when temperatures exceed 21°C (Kevrekidis et al., 2023). In the Iskenderun (Alexandretta) Bay in Turkey, the highest number of ovigerous females was recorded in July and August, while in the Beymelek Lagoon, also in Turkey, an 8-month spawning period was observed. In this lagoon, ovigerous females first appeared in February, peaking between August and September, with the spawning season lasting from July to September (Kevrekidis et al., 2023; Sumer et al., 2013). In North America, the reproductive period varies according

to latitude: from June to August in New Jersey, from May to September in Chesapeake Bay, from March to September in North and South Carolina, and from February to October in Florida (Kennedy et al., 2007). Females that release embryos in early spring are likely those that stored sperm from the previous summer (Kevrekidis et al., 2023).

Most females were found offshore, while the number of females found inshore was low. This reflects the typical pattern observed in their native range during the reproductive period, where females migrate offshore following their single mating event (Epifanio, 2019; Kevrekidis et al., 2023). The opposite trend was observed in males, as they are more commonly found inshore, consistent with observations from the western Atlantic coast, where males do not undergo the same migratory behavior as females (Aguilar et al., 2004). However, migration among some males has been documented in our study in the central Adriatic Sea; specifically, the few males observed offshore were all larger individuals, exceeding 150g. This suggests that only males of substantial size might be capable of undertaking such migration.

Migration among some males has also been observed by Kevrekidis et al. (2023) in the Northeast Aegean Sea, in the Eastern Mediterranean. In this study as well, the researchers observed that a small portion of males migrate in the open sea mainly from August to October, rather than within the upper lagoon.

In the Evros River estuary, a dominance of males over females was observed, while females were more prevalent in the adjacent coastal areas. The male dominance varied across different periods, with a ratio of 4:1 (males to females) in winter and spring, decreasing to 1-2.5:1 in summer and autumn, except for August, when male dominance peaked at a ratio of 8:1. In some parts of the lagoon, the sex ratio was equivalent in June, likely due to the onset of mating (Kevrekidis et al., 2023).

In Chesapeake Bay, males usually dominate the sex ratio in low salinity waters, while females dominate in high salinity waters, following their migration offshore (Guillory et al., 1996). Male dominance in lagoon areas has been documented in various regions, not only along the Atlantic coast but also within the Mediterranean basin, including Italian, Croatian, and Egyptian lagoons. However, in coastal areas, females dominate males from April to October, with ratios ranging from 1:3 to 1:9. (Kevrekidis et al., 2023).

There are no significant differences in the biometrics between inshore and offshore females; however, the average value is consistently higher in offshore females. This observation might be explained by the fact that females migrate offshore after mating, reaching sexual maturity and attaining their maximum size. Conversely, inshore females are often immature, as confirmed by histological analysis, and have not yet reached sexual maturity. Additionally,

some females that return inshore after the breeding season may contribute to the increased average size, as these individuals come back to the coastal areas to feed and prepare for the spawning season of the following year.

Studies conducted in multiple regions have documented the size at sexual maturity for female *Callinectes sapidus*, revealing a wide range of measurements depending on the specific regions. In the St. Johns River, Florida, the smallest recorded mature female measured 99 mm, while the largest immature female measured 177 mm (Tagatz et al., 1968). The estimated average size at sexual maturity for females in that region is between 150 and 160 mm (Sumer et al., 2013). In North Carolina, the smallest mature female was recorded at 52 mm, while the estimated average size at sexual maturity is 129 mm (Sumer et al., 2013 and Tagatz et al., 1968). In the Hudson River, immature females ranged in size from 10 to 95 mm, and mature females ranged from 95 to 125 mm, with a mean size of 109 mm for mature females (Chenery, 2002). In Chesapeake Bay, the smallest recorded mature female was 118 mm, and the largest immature female was 135 mm (Tagatz et al., 1968). The size range for mature females in this area is between 52 and 207 mm (Kennedy et al., 2007). Additionally, 50% of females at 132 mm were considered mature. The average size at sexual maturity is estimated at 144 mm, with other studies reporting estimates of 147 mm, 120 mm, and 132 mm (Chenery, 2002; Kennedy

et al., 2007 and Sumer et al., 2013). In Beymelek Lagoon, Southwestern Coast of Turkey, the size at sexual maturity for female crabs was estimated at 118.5 mm. In Tampa Bay, Florida, the size at sexual maturity was estimated at 130 mm. In Babitonga Bay, Southwestern Atlantic, the estimated size at sexual maturity was 102 mm. On the Southeast coast of Brazil, the size at sexual maturity was estimated at 103.3 mm (Sumer et al., 2013).

These data indicate significant variability in the size at sexual maturity for female *C. sapidus* across different geographic regions, with average sizes ranging from approximately 100 mm to over 160 mm.

Larger values are also observed in offshore males compared to inshore males. This could be attributed to the fact that larger males might better withstand the migratory effort. Kevrekidis et al. (2023) also reported smaller sizes in individuals captured in the lagoon compared to those offshore, which supports our findings. According to the researchers, this size difference in their study area is due to both increased fishing pressure in the lagoon and migratory movements related to spawning or overwintering.

Distinguishing both females and males across the three size classes, we observe that gonad weight and carapace dimensions (CL and CW) increase with size class. However, the general trend described previously remains consistent. In females, significant differences in both CL and CW are noted between inshore

and offshore individuals within the <100g size class. This is likely because inshore individuals are still sexually immature; they exhibit very reduced gonad weight, and their carapace dimensions are also smaller as they have not yet undergone the final molt that occurs prior to mating. Overall, there is considerable variability in size among mature females, particularly those migrating offshore.

In inshore males, we observe that biometric values increase with the individual's weight. However, for offshore males, data is available only for the highest weight class. Significant differences in carapace length were found between inshore and offshore males over 150g.

These findings, particularly regarding the weight of the gonads, need to be confirmed with a larger sample size, especially for the male specimens.

The temporal trend of offshore females' total weight, CL (carapace length), CW (carapace width) and GSI was then analyzed to understand whether there was a relationship between the sampling period and the size of different individuals. The value of total weight is the most variable, as there are offshore females with a weight greater than 170g and others with a weight of 110g, but it does not show a trend over time that can be attributed to a specific reproductive strategy, e.g., small females migrate offshore first and then large ones or vice versa. In contrast, regarding CW and CL values, these are more stable over time

and do not show much variability, so again no change over time is observed. As for the GSI, the highest value was recorded in September, followed by progressively lower values. This is consistent with data from other areas, where a reproductive peak is observed in September. A statistically significant difference was observed between the GSI of the samples collected on the first day of sampling and the peak value, as well as between the peak value and the final recorded value.

Analyzing the Gonad Somatic Index (GSI), it was found to be higher in offshore females compared to inshore females. When categorized by weight classes, the GSI is higher in offshore females within the <100g and 100g–150g size classes because they migrate offshore to reproduce, whereas it is lower in inshore females within the >150g size class. This difference may be attributed to the fact that larger inshore females are those that return after spawning. In males, however, the GSI is higher inshore compared to offshore and is greater in those weighing <100g compared to those >150g. This suggests that males likely undergo migration to overwinter rather than to reproduce. Consequently, after mating near the coast, with their testes emptied, they move offshore to spend the winter, resulting in a lower ratio between gonad weight and body weight in offshore individuals.

This is the first study in the Mediterranean Sea, specifically in the Adriatic Sea, to consider histological analysis of gonads of *Callinectes sapidus*. Until now, only macroscopic characteristics have been examined, based on the macroscopic classification by Hard (1942). No females with embryos were found inshore. The stages of gonad maturation observed inshore are consistent with the life cycle, showing immature and post-spawning individuals, with a small percentage in late secondary growth. It is typical to find immature females inshore because these coastal areas, often including estuaries, serve as juvenile habitats for blue crabs before they reach sexual maturity. Only females that have undergone mating, migrate offshore to develop their embryos and release larvae, which explains the absence of embryos among inshore females. Following the breeding season, some females may return to coastal areas to feed and prepare for the next spawning season. This aspect of their reproductive strategy, also observed in the Atlantic, explains the presence of post-spawning females inshore, as these individuals return to coastal areas to recuperate and prepare for the subsequent breeding season (Epifanio, 2019; Kennedy et al., 2007).

Similarly, in the study by Kevrekidis et al. (2023) conducted in the Northeast Aegean Sea, Eastern Mediterranean, ovigerous females were found exclusively in the coastal zone adjacent to the study area and not in the lagoon. However,

after the study period, some females with embryos were subsequently found inside the lagoon.

In the case of offshore females, observations in this study reveal individuals at various developmental stages, ranging from late primary growth (lPG) to post-spawning (PS). Embryos are present in females across different stages, including those in the secondary growth (SG) phase, reinforcing the idea that these females undergo multiple spawning events while offshore. Despite the presence of embryos, these females have gonads that are ready, or nearly ready, for the release of a new brood. This pattern has also been observed in several studies conducted in the Atlantic.

Female crabs exhibit a remarkable reproductive behavior, often spawning multiple times within the same season and across different years. Churchill (1919) observed that female crabs with embryos has fully developed gonads, so ready for subsequent egg release, thus they can undergo multiple spawning events within a single season as well as in the following season. These findings align with experiments indicating that female crabs may spawn twice or more over the course of their lives. Similarly, Harris (1982) noted that females that spawn in late spring might spawn again later in the same year and potentially once more the following spring.

Millikin et al. (1984) also supports the strategy of multiple spawning events, as does Van Engel (1958), who notes that female crabs may spawn two or more times during their lives. Darnell et al. (2009) further explains that spawning females produce multiple clutches and engage in migratory behavior between clutches, ensuring continuous movement seaward throughout the spawning season. This repeated spawning behavior is critical for the reproductive success and population maintenance of these species.

CHAPTER VI

CONCLUSION

This study revealed for the first time that *Callinectes sapidus* reproduces in the Adriatic Sea, particularly in areas where it was caught by local fishermen, as supported by the discovery of egg-bearing females. The reproductive strategy of the blue crab in the Adriatic is similar to that observed in the Atlantic and Mediterranean, with only minor differences. A key aspect of this research was the histological study, marking the first in-depth analysis of the reproductive biology of this species in the Adriatic. The histological approach confirmed the importance of the Gonadosomatic Index (GSI) as a bioindicator for monitoring this species. However, histology proved fundamental not only in distinguishing between the beginning and end of the reproductive season, but also in revealing that post-spawning females return inshore to reproduce again in the following season, a distinction that could not be achieved using only the GSI. Additionally, this pilot study identified the Adriatic as a spawning area that overlaps with fishing zones, highlighting a significant interaction between the species' reproductive behavior and fishing activities. These findings are important for future management of this invasive species, as they provide key insights into how it reproduces and its interactions with fisheries, which are critical for devising effective control and conservation strategies.

CHAPTER VII

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