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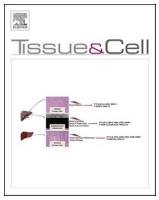

Histological-based characterization of ovarian developmental stages in blue crab (*Callinectes sapidus*) within the spawning season in the central western Adriatic Sea

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ABSTRACT

The Atlantic blue crab (*Callinectes sapidus* Rathbun, 1896) is a euryhaline and eurythermal species native to the Atlantic coasts of the Americas. Although its widespread distribution across the Mediterranean basin is well documented, information on its reproductive patterns remains limited. This study focused on the combination of both macroscopic and histologic characterization of ovarian developmental stages in female blue crabs along the north-central Italian Adriatic coast, within the spawning period. Samples were collected off the coast of Ancona, in collaboration with local fishermen, between September and November 2023. For each specimen, sex and biometric parameters were recorded to determine the sex ratio, while size distribution and gonadosomatic index (GSI) were calculated for female specimens. Ovary samples and eggs sponge were collected to determine the ovarian developmental stage and fecundity respectively. Results showed females as the dominant sex throughout the sampling period, with males being extremely rare. The collapse of female catches in early November, alongside the consistent presence of ovigerous females and the strong female-biased sex ratio, identified this area as a spawning ground. These findings align with reproductive patterns observed in the species' native range and other Mediterranean areas. Moreover, GSI values and fecundity indexes associated with macroscopic and histological examination of ovarian stages suggested that the end of the reproductive season corresponded to mid-November.

1. Introduction

The Atlantic blue crab (*Callinectes sapidus*, Rathbun, 1896) is a marine species of the Portunidae family, native to the north and south-western Atlantic coasts, inhabiting estuaries, tidal rivers, and shallow coastal waters (Mancinelli et al., 2021).

It is one of the numerous alien species that have colonized the Mediterranean basin and established prosperous populations over the years (Galil, 2011). Various biological traits give it the potential to successfully colonize non-native environments, including omnivorous feeding habits, aggressive behavior, high mobility, large body size, and high fecundity (Nehring, 2011). In particular, the high number of eggs

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produced per individual in each clutch (ranging from 284 thousand to 2.2 million) along with the flexibility of the blue crab's reproductive strategy in terms of timing and environmental adaptability, played a crucial role in its successful colonization of the Mediterranean area (Vivas et al., 2025).

To gain a deeper understanding of blue crab population dynamics, various studies (Kevrekidis et al., 2023; Kevrekidis et al., 2024; Türeli et al., 2018) conducted across the Mediterranean basin have identified spatial and temporal patterns of spawning based on the presence of ovigerous and non-ovigerous females, and by performing macroscopic evaluations of ovaries using the five-stage classification proposed by Hard in 1942. This classification distinguishes ovarian stages based on the color and size of the ovary, as well as the presence of the egg sponge.

Although this method is still widely used, macroscopic examination of the ovary in marine species can be subject to operator-related bias due to the challenges in clearly recognizing specific morphological traits in both vertebrate and invertebrate species (Chemello et al., 2023; Colella et al., 2024). This can be particularly challenging when identifying immature or post-spawning specimens or when distinguishing between different color grades. For example, between stages III and IV, where ovary appearance alone is insufficient to differentiate the two stages, the only reliable feature is the presence of egg sponge or eggshell remnants on the swimmerets (Hard, 1942). Histological analysis represents a valid tool to support the macroscopic evaluation of gonadal stages, especially for species that show significant variability in the timing of the reproductive period and the remarkable ability to reproduce in different environments, such as the blue crab. In the Adriatic Sea, where the presence of blue crabs has been established from north to south (Onofri et al., 2008; Cilenti et al., 2015; Manfrin et al., 2016; Mancinelli et al., 2024; Chiesa et al., 2025), information on the spawning period across different areas is still lacking.

To date, gonadal characterization based on microscopic analysis has only been performed on 15 female specimens collected from the Gargano Lagoon (southwestern Adriatic Sea) between 2013 and 2014 (Cilenti et al., 2015). To implement the understanding of blue crab reproduction in the Adriatic basin, spawning patterns must be investigated across multiple areas.

The present study characterized, for the first time, the female gonadal stages within the spawning period thanks to both macroscopic and histological analysis of a large number of specimens collected within a key fishing area of the north-central Adriatic Sea. The sampling effort, in terms of both specimen number and spatial coverage, offers a representative although preliminary overview of reproductive dynamics in a specific area of the north-central western Adriatic basin.

2. Materials and methods

2.1. Sampling

A total of 193 (189 females and 4 males) specimens of blue crab were accidentally caught using the rapido trawl in collaboration with local fishermen from the Ancona fleet (Italy) between September and November 2023 off the coast of the northern-central Adriatic Sea (FAO-GSA 17; coordinates: 43.63708, 13.69538) with multiple hauls per month at a depth of approximately 65 m. At each sampling time, the sex of each crab was macroscopically determined based on the different shapes of the abdomen between males and females. Crabs were individually weighted, and the carapace length (CL) and width (CW) were recorded. All the ovaries were also sampled, weighed, and macroscopically examined to determine the developmental stage. A portion of the ovary was collected from a subsample of females (N=100) and stored in a formaldehyde/glutaraldehyde solution (NaH_2PO_4–H_2O + NaOH + Formaldehyde (36.5 %) + Glutaraldehyde (25 %) + H_2O) for the subsequent histological analysis. From ovigerous females (N=94) as well, the mass of fertilized eggs was weighed separately after carefully removing the pleopod structure from the egg mass.

2.2. Sex ratio and gonadosomatic index

Total and monthly sex ratios were calculated with the formula: $f/(m+f)$.

The gonadosomatic index (GSI) was calculated for each female specimen using the following formula: $(GW/TW)\ x\ 100$, where GW and TW are the gonadal and total weight, respectively, expressed in g.

2.3. Histological analysis

Histological analysis was performed on a portion of each ovary as briefly described below (Conti et al., 2024). Tissues, stored in the fixative solution, were washed in ethanol 70 % and successively dehydrated in ethanol series at increasing concentrations (70 %, 80 %, 95 %, and 100 %). After dehydration, each ovary portion was individually embedded in paraffin. Sections of 4 μm were cut with a microtome (Leica RM2125 RTS, Nussloch, Germany), stained with Mayer's hematoxylin and eosin stain, and examined under an optical microscope (Zeiss Axio Imager.A2, Oberkochen, Germany). Images were acquired through a combined color digital camera Axiocam 503 (Zeiss, Oberkochen, Germany).

2.4. Macroscopic and microscopic evaluation of ovarian stages

Macroscopic evaluation of different gonadal stages was performed following the structural and morphological characterization of the ovary described by Hard W. L., (1942) and summarized in Table 1.

This study identified eight different ovarian stages, based on the histological criteria described by Brown (2009) (Table 2). Different germinal cell stages were described based on their structure, cytoplasmic content, and position.

2.5. Fecundity analysis

Fecundity was assessed following Kevrekidis et al. (2024). Briefly, the wet weight of egg masses attached to ovigerous females was recorded with high precision (± 0.001 g) after removing the pleopods. Four subsamples from each female (0.035±0.06 g average weight) were also weighed to the nearest 0.001 g, and the number of eggs in each was counted using a Bogorov chamber. These values were used to calculate the total egg count per individual. The egg mass index (EMI) was calculated with the following formula:

egg *mass weight (g) / total body weight (g) x 100*

2.6. Statistical analysis

All biometric data, GSI, fecundity, and sex ratio were analyzed using the GraphPad Software Prism8 for Windows. Biometric data and GSI values were analyzed through one-way ANOVA analysis after assessing the data's normal distribution with the Shapiro-Wilk test. Pairwise comparisons between group means were performed using Tukey's post

Table 1
Macroscopic criteria used to determine the ovarian developmental stages (I-V).

Stages	Description
immature	Small, pinkish filaments with small, thin, and white seminal receptacles.
Stage I	Immediately after the last molt, small white ovary.
Stage II	The ovary is orange and increases in length and diameter.
Stage III	Mature ovary preceding the first ovulation, ovaries are bright orange and large, female with no egg sponge.
Stage IV	Period between the first and second ovulation, the ovaries remain orange, egg sponge is present.
Stage V	The ovary has collapsed, grey or brown, egg sponge is still present on the swimmerets.

Table 2
Ovarian developmental stages based on histological analysis of the ovary.

Stages	Description
immature	Ovary with oogonia only, or oocytes in the preprimary growth stage.
***ePG** early primary growth*	The ovary presents a few clear cells similar to oogonia, with augmentation of the ooplasm for the formation of organelles.
***lPG** late primary growth*	Ovary with oogonia in the germinal zone are the predominant stages, oocytes in late primary growth with a perinuclear yolk nucleus complex. Oil droplets and cortical alveoli begin to form.
***eSG** early secondary growth*	Early formation of yolk globules of small volume.
***mSG** mid secondary growth*	Yolk globules continue to form and increase in size; the germinal zone is visible.
***lSG** late secondary growth*	Large yolk globules reach the maximum diameter; the germinal zone is less evident. Oocytes appear fully yolked.
***FG** full growth*	Yolk globules are fused; fully grown oocytes are located mainly on the perimeter of the ovarian lobe.
Post spawning	Ovaries in post-spawning present atretic oocytes. Absence of the germinal zone at the end of the reproductive season.

hoc test. The significance of the sex ratio deviation from 1:1 was calculated using Fisher's exact test. Data were reported as mean values ± SD. All the significances were set at $p \leq 0.05$.

3. Results

3.1. Sex ratio, biometric parameters, and GSI

Considering the total amount of crabs sampled between September and November, the total sex ratio was 0.98, which significantly differed from the expected values ($X^2 = 115$, df = 1, $p < 0.0001$) (Fig. 1A). Of all the females sampled, 49.7 % were ovigerous females while 50.3 % did not carry eggs (Fig. 1B). The monthly sex ratio showed a predominance of females in September and October, with a sex ratio of 0.95 and 0.99, respectively ($X^2 = 30.99$, df = 1, $p < 0.0001$ and $X^2 = 81.06$, df = 1, $p < 0.0001$, respectively) (Fig. 1A). In November, the sex ratio was not calculated since all animals sampled were females. Concerning the ratio of ovigerous and non-ovigerous females, no evident time-dependent trend of one category over the other was observed (Fig. 1B).

Analysis of biometric parameters did not evidence any significant differences in females' carapace length, width, and total body weight among sampling months (Fig. 2A, B, and C). Differently, GSI mean values were significantly lower in October and November when compared to September (Fig. 2D).

3.2. Macroscopic characterization of ovarian stages

The macroscopic evaluation of the ovaries revealed the presence of all five stages in September and October, while stages I and II were not detected in November (Fig. 3A), and the absence of immature specimens. Females that had a stage III ovary were characterized by a significantly higher mean value of GSI when compared to the other stages (Fig. 3B).

3.3. Histological analysis

Neither immature nor in early primary growth (ePG) females were identified through examination of the histological images. All stages reported in Fig. 3 were observed among all samples analyzed.

Ovaries in late primary growth (lPG) presented a basophilic ooplasm, which stained blue violet with Hematoxylin and Eosin stain (Fig. 4A). In lPG ovaries, the perinuclear yolk complex (PYC) is visible within the oocyte as well as the oil droplets (od) (Fig. 4B). The subsequent stage of early secondary growth (eSG) is characterized by an ooplasm less basophilic, where oil droplets (od) and first yolk globules (yg) can be observed (Fig. 4C and D). At this stage, the germinal zone (GZ) is easily identifiable (Fig. 4D). In mid-secondary growth (mSG), the acidophilic ooplasm is stained pink, the number of yolk globules increases, and the GZ is still evident while oil droplets are no longer visible (Fig. 4E and F). The number and size of yolk globules further increased in late secondary growth oocytes (lSG), occupying almost the entire volume of the oocyte (Fig. 4G and H). At this stage, the GZ is less evident (Fig. 4G). Finally, the yolk globules begin to fuse from the periphery of the oocyte towards the center, giving the ooplasm a more homogeneous appearance typical of an ovary in the full-growth stage (FG) (Fig. 4I and J). Some of the female specimens analyzed showed one of the two typical ovarian structures of the post-spawning phase. In females that were potentially able to spawn a new batch of eggs, the germinal zone was visible again, and they presented eSG oocytes (Fig. 4K). Differently, females that couldn't perform a new spawning event presented ovaries with no germinal components and diffuse extracellular matrix (Fig. 4L).

The presence of different ovarian stages across sampling months did not exhibit any time-dependent trends, except for post-spawning females, which increased in number over the months (Fig. 5A). The greatest variability in stages was observed in September, followed by October. In November, only females at the post-spawning and mSG stages were observed (Fig. 5A). All females at the lSG stage were non-ovigerous, while all females observed in eSG and mSG were ovigerous females (Fig. 5B). Differently, specimens identified as lSG, full-growth, and post-spawning included both non-ovigerous and ovigerous females.

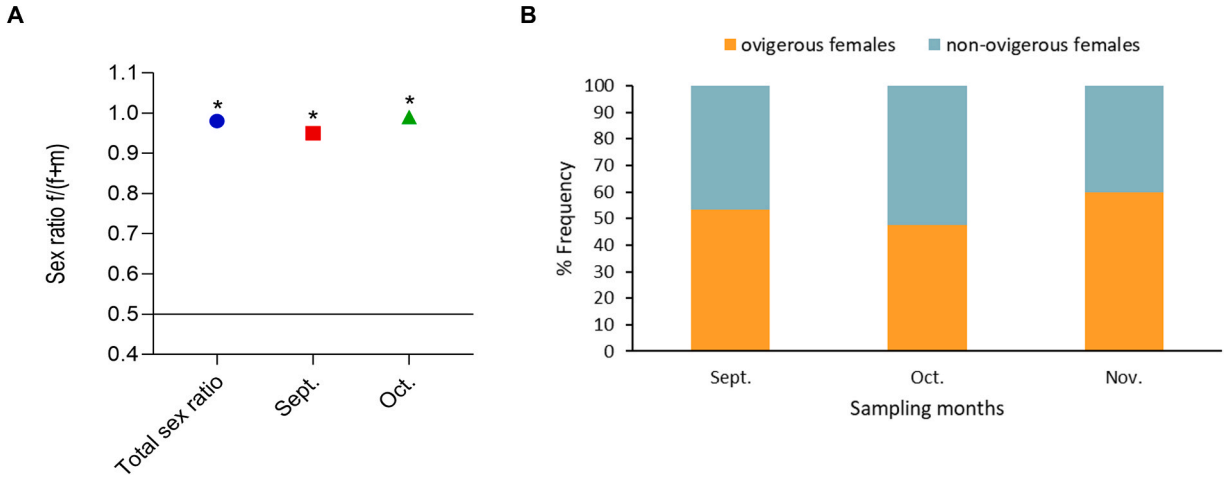

Fig. 1. Sex ratio. Total and monthly sex ratio (A). * Indicates sex ratio value with a statistically significant ($p < 0.05$) deviation from 1:1 calculated using Fisher's exact test; frequency (%) of ovigerous and non-ovigerous females within each sampling month (B).

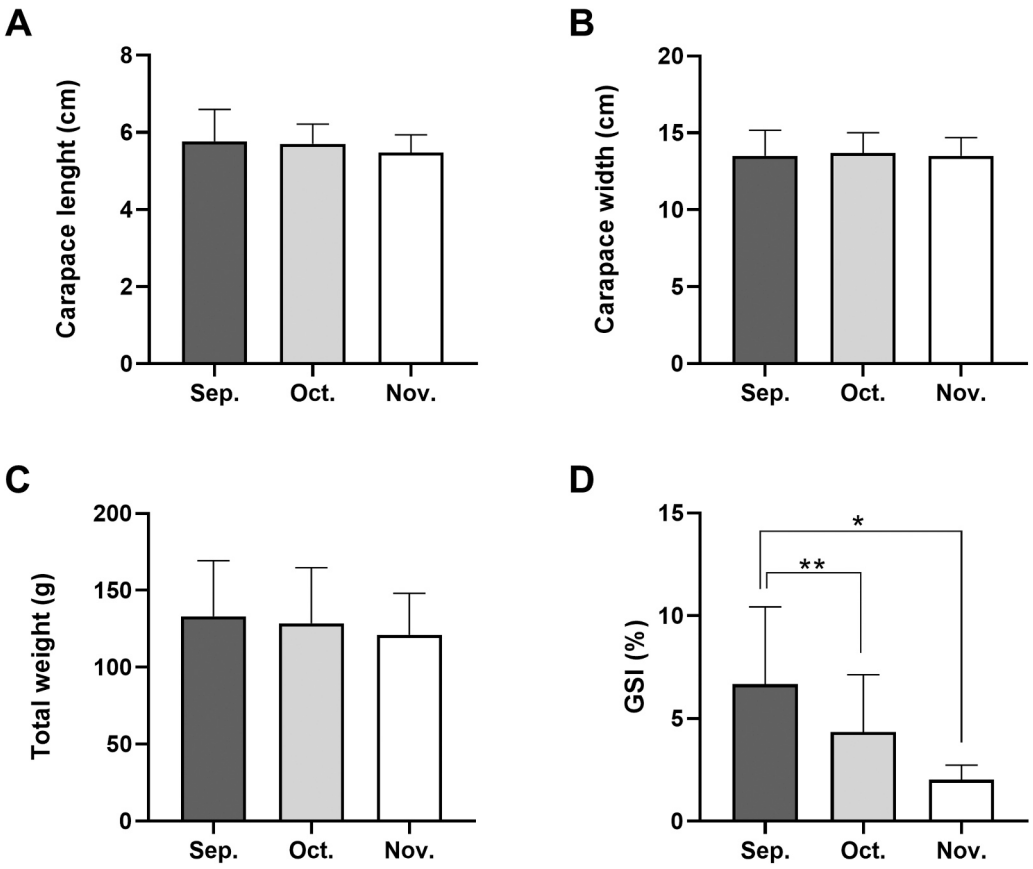

Fig. 2. Biometries and GSI. Female blue crab's carapace length (cm) (A) and carapace width (cm) (B), weight (g) (C), and gonadosomatic index (GSI %) (D), among sampling months. Data are expressed as mean value ± standard deviation. Significance was set at *$p \leq 0.005$, and ** $p \leq 0.001$.

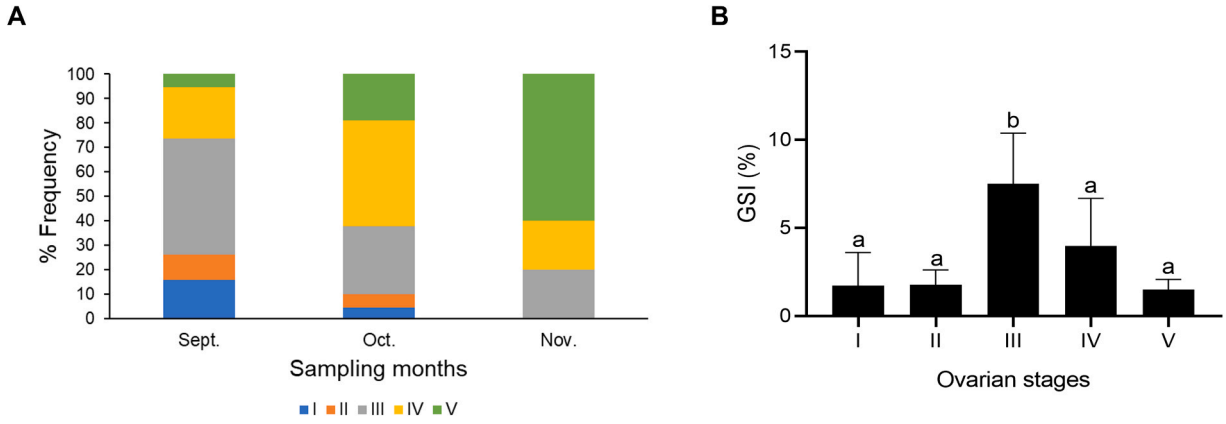

Fig. 3. Macroscopic ovarian stages and GSI Frequency (%) of ovarian macroscopic ovarian stages (I-V) among sampling months based on macroscopic evaluation of the ovary (A); GSI (%) values among different macroscopic ovarian stages (B), data are expressed as mean values ± standard deviation; different letters indicate significant differences ($p \leq 0.05$).

GSI values in different ovarian developmental stages increased following steps of oocyte development and maturation, with the highest values at the lSG and full-growth oocyte stages (Fig. 6) that were significantly different compared to all the other stages. Only one specimen was observed in early primary growth (ePG) during the whole sampling period.

3.4. Fecundity analysis

Both EMI (Fig. 7A) and absolute egg count (Fig. 7B) showed a significant decreasing trend across the sampling months. EMI and egg count values were significantly lower in October and November when compared to September's mean values ($p < 0.001$ and $p < 0.01$ respeectively for EMI, and $p < 0.001$ for the eggs number) (Fig. 7A, B).

4. Discussion

Despite growing concern over blue crabs' widespread presence in the Mediterranean Sea, a significant knowledge gap persists regarding their reproductive strategy in several areas, including the Adriatic region. To date, only a single year-long study focused on the presence and population dynamics of blue crabs in the Southwestern Adriatic Sea within

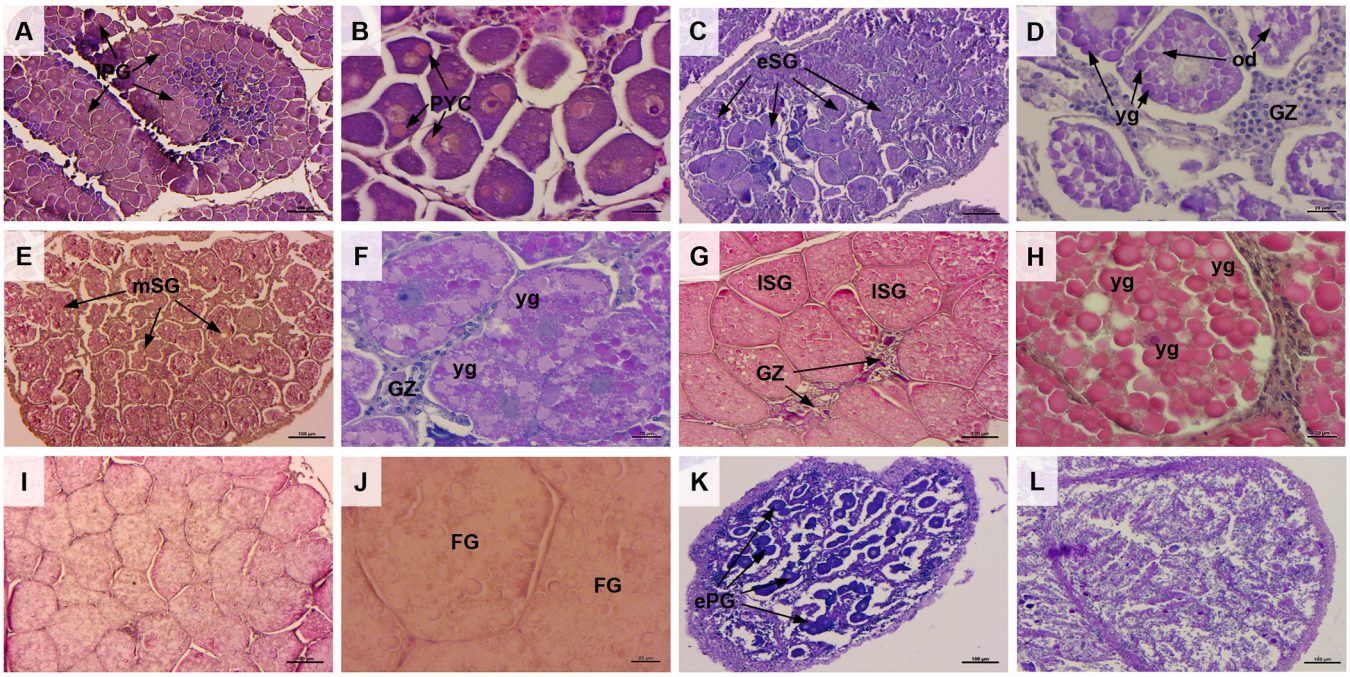

Fig. 4. Representative histological images of different ovarian developmental stages. A and B, late primary growth (lPG); C and D, early secondary growth (eSG); E and F, mid-secondary growth (mSG); G and H, late secondary growth (lSG); I and J, full growth (FG); K, post-spawning ovary with germinal zone; L, post-spawning ovary with no germinal zone. PYC, perinuclear yolk complex; yg, yolk globules; od, oil droplet; GZ, germinal zone. Scale bars: 100 μm and 20 μm for A, C, E, G, I, K, L and B, D, F, H, J, respectively.

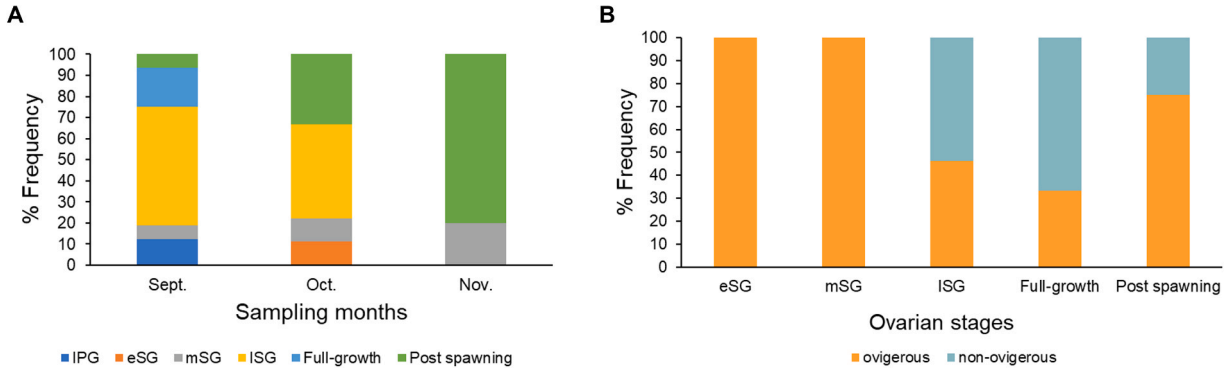

Fig. 5. Microscopic ovarian stages. Frequency (%) of microscopic ovarian developmental stages among sampling months based on histological analysis of the ovary (A); frequency (%) of ovigerous and non-ovigerous females within each developmental stage (B). lPG, late primary growth oocyte; eSG, early secondary growth oocyte; mSG, mid secondary growth oocyte; lSG, late secondary growth.

the Lesina Lagoon (Mancinelli et al., 2024). However, the unique hydrodynamic characteristics and shallow brackish waters of this environment create a distinct niche that does not represent the entire Adriatic basin.

In the present study, data collection focused on specimens from a specific fishing area (located in the offshore waters of the Ancona coast) where samplings were conducted during three different months. Samples collection in August was not possible due to the fishing ban in the northern central Adriatic Sea, which lasted until mid-September. In contrast, samplings stopped at the beginning of November due to the complete drop in blue crab catches within the sampling area. It should be noted that this limited sampling period also depended on the total absence of blue crab specimens in the sampling area during the months preceding and following this time range, as daily monitoring of fishing catches confirmed.

The predominance of female specimens, throughout the entire sampling period (only 4 males out of 193 crabs), along with the consistent presence of ovigerous females, identified the study area as a spawning ground for blue crabs in the Adriatic Sea. Furthermore, the presence of only mature females, with no significant differences in size, supports the hypothesis that their migration offshore towards this area occurs solely for spawning purposes. This spawning behavior is well documented in the native range of the blue crab, where females typically undertake offshore migrations following a single mating event during the reproductive period, while males do not exhibit the same migratory pattern (Epifanio, 2019; Kevrekidis et al., 2023; Aguilar et al., 2005). The occurrence, although minimal, of males in the sampling area offshore did not appear to be associated with any specific factor; therefore authors did not speculate on this aspect, considering the scarce information available.

This could be related to a form of sperm-guarding behavior, involving a prolongation of the typical post-copulatory guarding of the female (McLay and Becker, 2015). Alternatively, since all the sampled 'migratory' males exceeded 150 g in total weight, the migration may

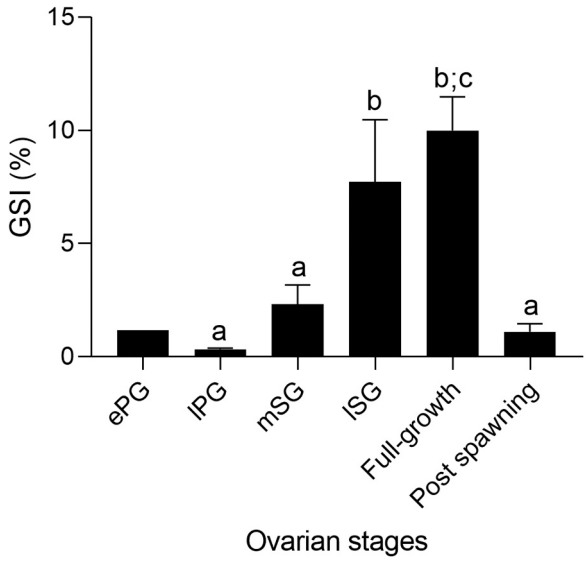

Fig. 6. GSI. GSI (%) values among different ovarian stages; data are presented as mean values ± standard deviation, except for the ePG group. Different letters indicate significant differences ($p \leq 0.05$).

represent a size-dependent behavior. Concerning the beginning of the spawning season, although no samples were collected in August, it is possible that spawning had already begun by that period.

The decreasing trend in the GSI also supported this hypothesis, suggesting a possible spawning peak in September, when the highest mean values were recorded, followed by a decline in the subsequent spawning months. On the other hand, the absence of samples after early November may indicate both the end of the spawning period and the migration of females to alternative spawning grounds. However, the data obtained supported the first hypothesis, primarily based on the decreasing trend in GSI, and secondarily on the evaluation of the ovary. Indeed, both macroscopic and histological analyses revealed a progressive reduction in the occurrence of stages typical of the spawning peak (stages III, IV, and lSG, full-growth oocytes of macroscopic and histological characterization respectively) and throughout the sampling period, accompanied by a corresponding increase in the frequency of post-spawning ovaries (corresponding to stage V of Hardy's macroscopic classification). These stages are commonly identified as indicative of active spawning ovaries, a classification supported by the highest GSI values recorded in females at these stages. The increased frequency of stage V toward the end of the spawning season has previously been observed in female blue crabs from another Mediterranean area during the reproductive period (Kevrekidis et al., 2023; Kevrekidis et al., 2024). Similar to the GSI trend among the sampling months, fecundity indices such as EMI and total egg count also indicated the end of the spawning season in November. This reduction in egg production has already been observed both in Mediterranean and Atlantic populations (Kevrekidis et al., 2024; Darnell et al., 2009; Dickinson et al., 2006; Graham et al., 2012). These studies observed a decrease in fecundity with successive broods, as primiparous females at the beginning of the spawning season were the most fecund, while multiparous females (those with second or later broods) in the following months showed lower fecundity.

The results obtained in the present study confirmed a degree of plasticity in both the timing and duration of the blue crab's reproductive period among different regions of the Mediterranean. Indeed, while this study suggested that the spawning season could occur between August and November in the north-central western Adriatic, it differs from what was observed in the Lesina Lagoon, where it extended from April to August (Mancinelli et al., 2024). Regional variations have also been commonly observed outside the Adriatic area. Past studies observed that egg production generally begins earlier in the spring at lower latitudes (Kevrekidis et al., 2023). For instance, in Iskenderun Bay (Levantine Sea), ovigerous females have been most abundant during July and August, whereas in the Beymelek Lagoon (Levantine Sea), an extended spawning period of approximately eight months (from February to September) has been observed (Kevrekidis et al., 2023; Sumer et al., 2013). Similarly to what has been observed in the Mediterranean area, the blue crab's reproductive period varies with latitude in the North-West Atlantic as well. It extends from June to August in New Jersey, from late May to early August in the Chesapeake Bay, from May to November in North Carolina, and from March – May, August – October in Florida (Darnell et al., 2009; Hart et al., 2021; Schneider et al., 2024).

Given the considerable variability observed for this species, the precise identification of ovarian stages offers another valid method to accurately determine the timing and duration of the reproductive season. Regarding the methodologies applied in this study, discrepancies were observed in stage identification between macroscopic and microscopic analysis. While immature females (stage I) were recorded in September based on macroscopic assessment, histological analysis did not identify any immature individuals throughout the entire sampling period. These findings highlighted the importance of integrating both macroscopic and microscopic approaches, rather than relying solely on external gonadal features or the presence or absence of egg sponges. Moreover, to obtain a complete overview of the reproductive period, a

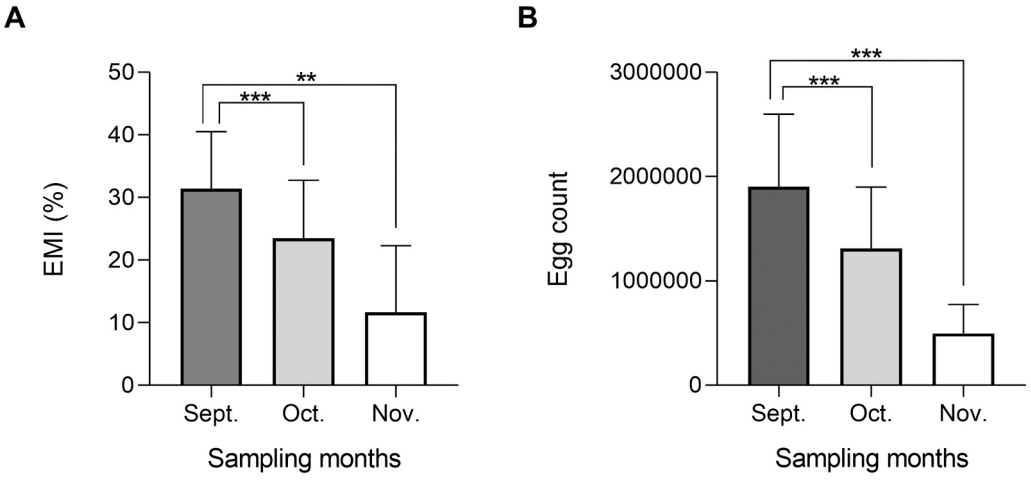

Fig. 7. Fecundity. Egg mass index (EMI %) (A) and egg count (B) among sampling months. Data are expressed as mean value ± standard deviation. Significance was set at ** $p \leq 0.01$ and ***$p \leq 0.001$.

sampling design including the months before and after the study range could be carried out to confirm the absence of specimens.

5. Conclusion

This study provided new insights into the reproduction of *Callinectes sapidus* in the north-central western Adriatic Sea, identifying an offshore spawning ground characterized by a spawning period that with high probability occurs from August/September to early November, with a likely peak in September. Discrepancies between macroscopic and histological ovarian staging emphasized the need for integrated methodologies to ensure accurate reproductive assessments. In particular, combined studies could facilitate monitoring the non-reproductive period and anticipating potential changes at the onset of the breeding season. Overall, this research contributed to a better understanding of blue crab reproductive strategies in the Adriatic and highlighted the importance of region-specific studies for effective monitoring of this species.

CRediT authorship contribution statement

Ike Olivotto: Writing – review & editing. **Giorgia Gioacchini:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Conceptualization. **Giulia Chemello:** Writing – original draft, Visualization, Validation, Investigation, Formal analysis, Conceptualization. **Loredana Manfra:** Writing – review & editing, Funding acquisition. **Giovanni Libralato:** Writing – review & editing. **Cecilia Silvestri:** Writing – review & editing, Investigation. **Ludovica Di Renzo:** Writing – review & editing, Investigation. **Laura Ciaralli:** Investigation. **Marco Matiddi:** Writing – review & editing, Investigation. **Erica Trotta:** Visualization, Investigation. **Elena Giannico:** Investigation, Formal analysis. **Matteo Zarantoniello:** Investigation, Formal analysis.

Ethical approval

In the present study, no ethical review and approval were requested for this study since the Italian legislation (D.L. 04/04/14 N.26) states that no ethical approval is required for experiments carried out on invertebrates.

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Declaration of Competing Interest

The authors report no declarations of interest.

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Data availability

Data will be made available on request.

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