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Dynamic regulation of apoptotic and antioxidant pathways throughout the reproductive cycle in female blue swimmer crab

Shabnam Adhami[1]*, Negin Salamat[1], Ahmad Savari[1], Gholamreza Hamidian[2]

[1] Department of Marine Biology, Faculty of Marine Science and Oceanography, Khorramshahr University of Marine Science and Technology, Khorramshahr, Iran; [2] Department of Basic Sciences, Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Iran.

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Abstract

This study investigated the antioxidant and apoptotic systems of blue swimmer crabs at various reproductive stages, providing valuable insights into their potential as biological markers, particularly in the polluted Persian Gulf. Our research along the coasts of Hendijan County, Iran, involved capturing live crabs (167 ± 52.07 g), examining their morphological traits and determining their reproductive stages through dissection and histological analysis. Apoptosis was detected using the TUNEL assay (terminal deoxynucleotidyl transferase dUTP nick end labeling), and enzyme activities including superoxide dismutase, catalase and glutathione peroxidase were measured using colorimetric methods. Variations were observed in the abundance of apoptotic cells within the hepatopancreas across reproductive stages. The second stage exhibited the lowest values and the first stage displayed the highest indicating a potential link between reproductive activity and apoptosis. Furthermore, enzymes representing the antioxidant system demonstrated various activities during ovarian development. Notably, the second ovarian stage demonstrated the highest catalase (5.63 mM *per* g protein) and malondialdehyde (12.14 mM *per* g protein) activities indicating an elevated response to oxidative stress. Our findings demonstrated that apoptotic cell numbers were fluctuated throughout the reproductive stages in the crabs, with the highest levels observed during the first stage and the lowest during the second stage. Understanding these fluctuations not only aids in distinguishing between reproductive and non-reproductive phases but also offers valuable insights into the broader physiological changes occurring throughout the cycle.

Introduction

Understanding crustacean reproductive biology is crucial for evaluating aquatic stocks, guiding conservation efforts and ensuring effective fisheries management. This is typically achieved through comprehensive data on reproductive periods, obtained via periodic population sampling.[1] Crabs, essential to the Persian Gulf ecosystem, thrive in diverse marine environments showing resilience and adaptability. Besides their ecological importance, they hold substantial economic value but are vulnerable to exploitation in marine stocks.[2] Exploring the reproductive organs of female blue swimmer crabs (*Portunus segnis*) in the Persian Gulf, specifically in the Bahrekan region, is vital due to the significant demand for their hepatopancreas and ovaries as edible delicacies.[3]

The Bahrekan region is particularly important as it is exposed to a wide range of pollutants and toxicants from oil and petroleum pollution including the Nowruz oil field. The area is also subject to international shipping, oil tanker transportation and other maritime activities, further emphasizing the need for thorough investigation.[4] Assessing apoptosis and the enzymatic antioxidant system during different reproductive stages provides valuable insights into the health and physiology of these crabs. Understanding the interactions between reproduction and endocrine systems is essential for comprehending the alterations in the hepatopancreas during reproductive processes.[5]

Reproduction is an energetically demanding activity that increases metabolic rates.[6] Vitellogenesis, regulated by reproductive regulators and signaling pathways, is a

***Correspondence:**

Shabnam Adhami. PhD
Department of Marine Biology, Faculty of Marine Science and Oceanography, Khorramshahr University of Marine Science and Technology, Khorramshahr, Iran
E-mail: sh.adhami@kmsu.ac.ir

complex process in crustaceans.[7] During ovarian development stages, the hepatopancreas serves as a site for cell biosynthesis.[8] As a digestive gland, the hepatopancreas is involved in digestion, nutrient absorption, storage, and excretion. Metabolic, histological and histochemical changes in the hepatopancreas occur in response to physiological needs like reproduction and digestion.[9]

The antioxidant defense system, which protects animals from damage caused by oxidative compounds, includes both enzymatic and non-enzymatic components. The hepato-pancreas, a key organ in synthesizing proteins and reflecting metabolic activities, plays a significant role in this defense.[10] Non-enzymatic antioxidant agents such as proteins are crucial for maintaining metabolic balance.[11] Various internal and external stimuli including those related to reproduction can trigger the production of reactive oxygen species (ROS) in crabs. The ROS are essential for initiating apoptosis, a process that helps eliminate defective cells and maintain overall organismal integrity.[12,13]

Recent studies underscore the intricate relationship between antioxidants and reproductive processes in crabs. Research has shown that antioxidant enzyme activity in the hepatopancreas of female crabs is hormonally regulated by estradiol and progesterone during vitellogenesis.[2] Additionally, natural water pollutants such as nitrogen compounds have been found to induce apoptosis and metabolic suppression, thereby, impacting antioxidant mechanisms.[6] Other studies have highlighted that ovarian growth is influenced by steroid hormone metabolism and that a diet rich in quality fats can enhance this process indicating that oxidative stress can be mitigated through dietary adjustments.[5] Also, carotenoids have been identified as effective modulators of oxidative stress.[12] Prolonged exposure to pollutants in contaminated environments can overwhelm the antioxidant defenses of crustaceans, exacerbating oxidative stress and apoptosis particularly in the hepatopancreas. Monitoring these responses is crucial for understanding the impact of toxicants.[14]

This study aimed to explore the reproductive stages of *P. segnis*, distinguishing between reproductive and non-reproductive phases. Significant changes in lymph protein content, antioxidant systems, and pollutant-induced alterations, such as apoptosis, throughout the reproductive cycle were monitored. By identifying the stages where apoptosis was most and least prevalent, the research provided crucial insights into the crab reproductive health and vulnerability to environmental stressors. This knowledge allowed for more targeted conservation strategies including timing protective measures to align with critical reproductive stages. Ultimately, this research had significant implications for managing and preserving the blue swimmer crab species in the Persian Gulf ensuring their survival in a changing environment.

Materials and Methods

Study area and experimental animals. This investigation was conducted along the coasts of Hendijan County, Iran, located in the southeastern part of Khuzestan province. The study area encompassed the Bahrekan wharf (30° 11’ 49.7”), situated in the northern region of the Persian Gulf. The elevation in this vicinity reached 5.00 m above sea level. Sampling in the Bahrekan fishing ground was conducted using a fishing vessel (Sambuk), capturing a specific number of female *P. segnis* crabs with a consistent average weight (a minimum of ten in each reproductive period) using a trawl net. Samples were collected from three distinct stations: Station I (Bahrekan fishing ground: 29° 56’ 575” N, 49° 28’ 476” E), Station II (Nowruz oil platforms: 29° 30’ 084” N, 49° 24’ 226” E) and Station III (Bahrekan oil platforms: 29° 52’ 008” N, 49° 26’ 294” E). Environmental factors including salinity, pH, temperature and dissolved oxygen were measured using a multimeter (Multi 340/SETi, Ankara, Turkiye). Sampling frequency was determined based on the reproductive and non-reproductive stages. Live crabs (at least 10 *per* reproductive stage) with an average weight of 167 ± 52.07 g were transported to the laboratory in a 100-liter fiberglass container filled with seawater equipped with an air pump, water filter and sample separator. Gender detection relied on morphological characteristics of the abdomen.[5] The animals were anesthetized by immersion in a clove powder solution (200 ppm),[3] following which biometric parameters including weight (accuracy: 0.01 g), length and width of the carapace were measured. Subsequently, the hepatopancreas and ovary were removed and weighed using a digital scale after dorsal and lateral cuts.

Immunohistochemical apoptosis assay. Immunohistochemical staining was performed using the TUNEL-POD (terminal deoxynucleotidyl transferase dUTP nick end labeling) kit from Abcam (Cambridge, UK)[15] to identify and assess apoptotic cells. The apoptotic index was determined by counting 20 fields in each group each containing at least 400 cells. Tissue sections of the hepatopancreas and ovary were prepared using conventional histological methods including dehydration, clarification and paraffin embedding utilizing the Histokinet rotary device (RX 11B; Tissue-Tek, Tokyo, Japan).[16] To perform the TUNEL test, the 3'-OH ends of DNA double strands in apoptotic cells were labeled using a nucleotide conjugated with biotin, facilitated by the terminal deoxynucleotidyl transferase enzyme (such as peroxidases or phosphatase). This process allowed the detection of DNA fragments damaged during apoptosis using immunohistochemical methods. The procedure was as follows: After standard preparation of paraffin sections and deparaffinization in two containers of xylene, followed by rehydration in decreasing concentrations of ethanol,

the slides were washed three times in phosphate-buffered saline. Next, proteinase K solution (20.00 µg mL^{-1} in buffer) was applied to the slides and the sections were incubated at 37.00 ˚C for 30 min. To prevent the TUNEL solution from reacting with endogenous proteins (internal peroxidases), the slides were treated with 0.30% hydrogen peroxide in methanol for 10 min. Following another buffer washing and drying, the sections were incubated with 50.00 µL of TUNEL reaction mixture (45.00 µL of labeled nucleotide solution and 5.00 µL of enzyme solution) at 37.00 ˚C for 60 min. After fluorescein-labeled nucleotides were attached, the sections were washed again and incubated with an anti-fluorescein antibody solution conjugated with peroxidase for 30 min at 37.00 ˚C. The sections were then washed with buffer and treated with 50.00 µL of diamino-benzidine solution (5.00 µL of diaminobenzidine solution in 45.00 µL of peroxidase buffer) at room temperature for 15 min to develop the color reaction. Finally, the sections were dehydrated in increasing concentrations of ethanol, cleared in two containers of xylene and mounted with coverslips. The slides were then examined under an optical microscope. Apoptotic cell nuclei exhibited a brown color. Additionally, tissue sections of the hepatopancreas and ovary were stained with Hematoxylin and Eosin for histometric study. Digital images were captured using an optical microscope and Dino Capture software (FDP2, New Taipei City, Taiwan).

Enzymatic assay. At this stage, hepatopancreas tissue (0.50 g) was homogenized with 5.00 mL of 0.40 M phosphate buffer (Sigma-Aldrich, St. Louis, USA) and promptly centrifuged for 10 min at 4.00 ˚C and 3,000 rpm. The resulting supernatant was stored at – 80.00 ˚C for subsequent analysis of antioxidant parameters using a spectrophotometric device (Lambdatm 750 UV/Vis/NIR; Perkin Elmer, Waltham, USA). The total protein content of the hemolymph was determined using the Biuret reaction method with optical intensity measured at 540 nm.[17] The superoxide dismutase (SOD) activity was measured by preparing a reagent with 50.00 mM Tris hydrochloride buffer (pH 2.80; Sigma-Aldrich) and 10.00 mM pyrogallol solution (pH 4.70; Sigma-Aldrich). After mixing the buffer with the homogenized liver sample, pyrogallol was added and SOD activity was assessed by monitoring the reduction in light absorption at 420 nm.[18] Catalase activity was determined using a mixture of 0.050 M Tris hydrochloride buffer (pH 8.70), 0.03% H_2O_2, (Iran Chemical Mine, Isfahan, Iran) and 4.00% ammonium molybdate (Anmol Chemicals, Mumbai, India), with reactions incubated at room temperature and measured its activity at 410 nm.[18] For glutathione peroxidase (GPx) activity, potassium phosphate buffer with pH 5.70 (Sigma-Aldrich), nicotinamide adenine dinucleotide phosphate (Sigma-Aldrich), reduced glutathione and glutathione reductase were combined and enzyme activity was measured at 340 nm after incubation at 37.00 ˚C. Malondialdehyde (MDA) activity, indicative of lipid peroxidation, was determined based on thiobarbituric acid inhibition using a spectrophotometer.[19]

Statistical analysis. Statistical grouping based on treatments involved categorizing the five distinct stages of ovarian development, determined by various criteria including morphological features such as color, size and gamete diameter alongside histopathological observations and histometric assays. Somatic indices such as hepatosomatic index (HSI) and gonadosomatic index (GSI) were also integral to this comprehensive assessment. Results were presented as mean ± standard deviation with each experiment replicated at least five times. Normality was assessed using the Shapiro-Wilk test and homogeneity of variances was checked using Levene's test. Upon confirmation of normal distribution, one-way analysis of variance and post-test Tukey were employed to compare data among different reproductive periods and apoptotic index changes across stages with the control group at a significance level of 0.05. Data analysis was conducted using SPSS Software (version 20.0; IBM Corp., Armonk, USA) with Excel (version 2013; Microsoft Corporation, Redmond, USA) utilized for graph preparation.

Results

Environmental factors. The average measured environmental factors in the water at the sampling stations were as follows: Temperature (28.50 ± 0.90 ˚C), salinity (38.40 ± 0.90 ppt), pH (8.06 ± 0.90), electrical conductivity (3,804 ± 181 µS cm^{-1}) and dissolved oxygen (7.60 ± 0.50 mg L^{-1}).

Morphological parameters. The biometric results for the *P. segnis*, encompassing parameters such as body weight, carapace width, carapace length and width of the last abdominal segment are illustrated in Figure 1. This figure also compares the average body weight across various stages. No significant difference was observed in the measured biometric parameters among different stages ($p > 0.05$). The examination of the GSI in female crabs revealed the lowest value at the initial reproductive stage (3.81) and the highest at the fourth stage (14.08). The GSI demonstrated a consistent upward trajectory throughout the diverse stages, reflecting the growth of ovaries and sexual maturation. Notably, the peak GSI values coincided with the commencement of the fifth stage, indicating impending spawning. Conversely, no substantial distinction in the HSI was observed across various ovarian stages. However, hepatopancreatic cells during reproductive phases exhibited characteristic changes, including shifts in nuclear density. Particularly in the latter stages, a notable decline in the integrity of both cells and their nuclei was evident.

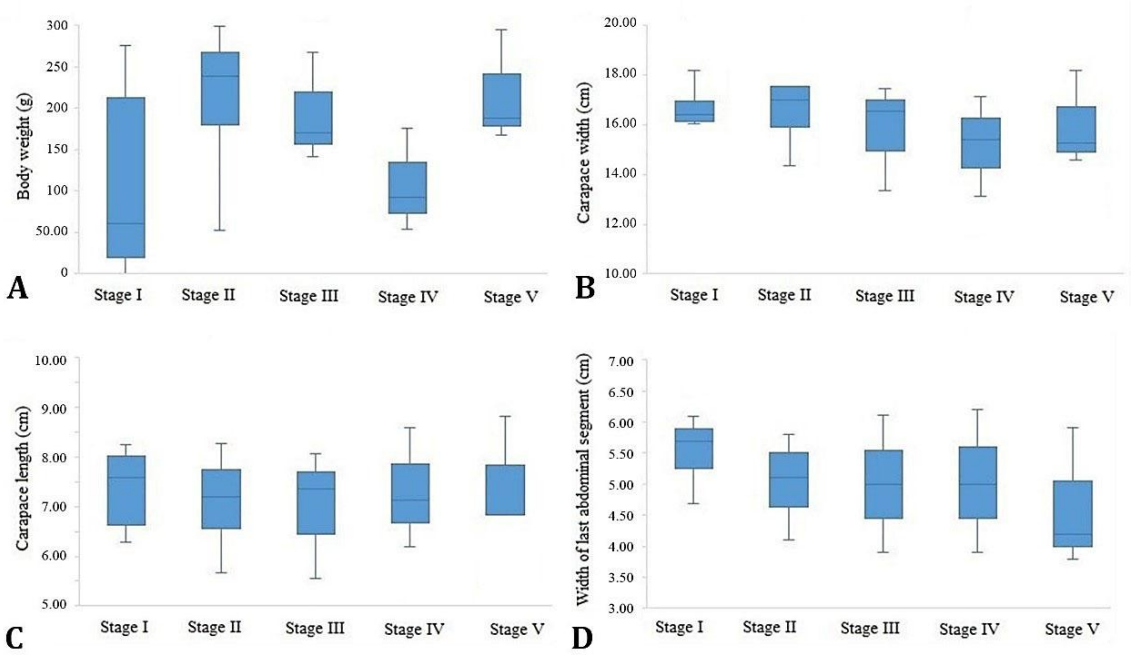

Fig. 1. A) Body weight, **B)** Carapace width, **C)** Carapace length, and **D)** Width of last abdominal segment of blue swimmer crab across various the reproductive stages. Data are presented as mean ± SE. No significant differences were observed among the groups ($p > 0.05$).

Hepatopancreas apoptosis. Microscopic images captured under a light microscope illustrated apoptotic cells within the tissue structure of the blue swimmer crab hepatopancreas during various reproductive stages. In Figure 2, positively stained (apoptotic) tunnels are characterized by golden-brown nuclei displaying a uniform and dense color. Conversely, negatively stained or pale nuclei represented negative test tunnels. The images were captured using a 20× objective lens. The second ovarian stage exhibited the lowest number of apoptotic cells, significantly fewer than other stages ($p < 0.01$; Fig. 3). Subsequently, in the third ovarian stage, the apoptosis index was increased significantly compared to the second stage ($p < 0.01$). The fourth ovarian stage displayed a significantly higher number of apoptotic cells than the second and third stages ($p < 0.01$). The first ovarian stage had the highest number of hepatopancreas apoptotic cells which did not show a statistically significant difference from the fifth ovarian stage ($p = 0.09$).

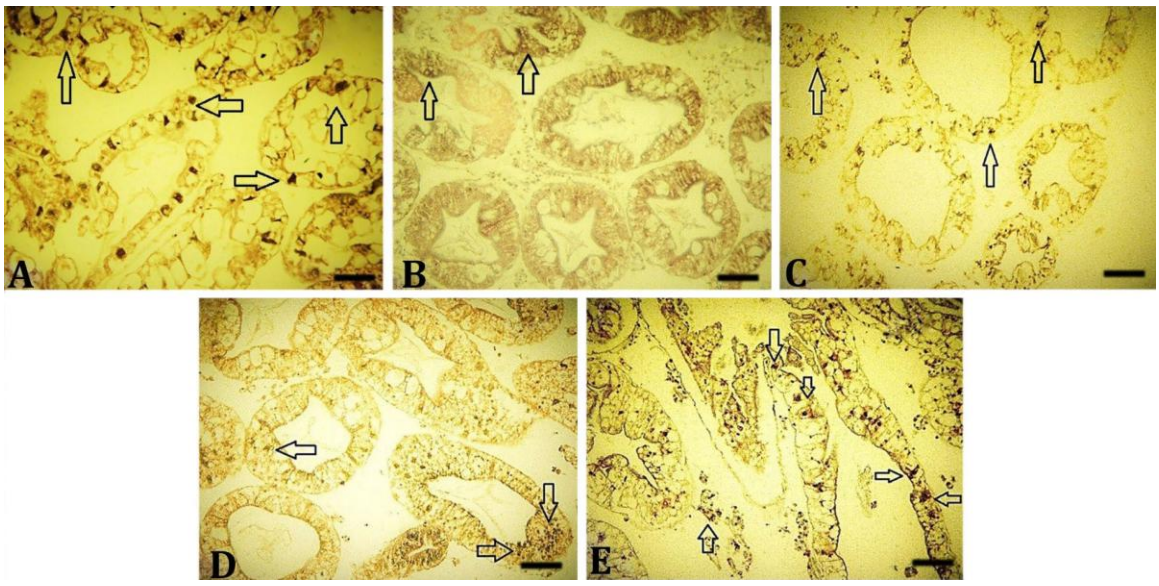

Fig. 2. The progression of apoptosis across various stages **A - E)** Stage I - V of gonadal development in female crabs (TUNEL assay, 725 ×). The hollow arrowsshow apoptotic cells.

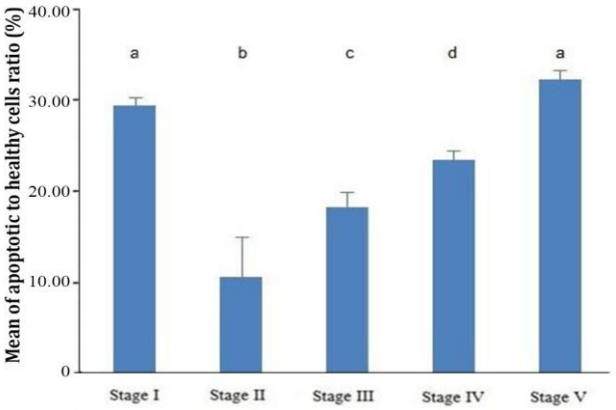

Fig. 3. Mean of apoptotic cells to healthy cells ratio (%) in blue swimmer crab across the various reproductive stages.
[a-d] Statistical differences among the groups are shown with different letters at $p < 0.05$.

Antioxidant responses. Figure 4 shows the results of this section, highlighting a decrease in enzymatic activity levels during female crab sexual development and maturation. While differences in total protein content, GPx and SOD activities were not statistically significant ($p > 0.05$; Fig. 4A, B and C). Catalase and MDA activities showed a noticeable distinction from the third stage of ovarian development. The average of stage two was significantly different compared to stages three, four and five, but not to that of the first stage (Figs. 4D and E, $p < 0.05$).

Discussion

In invertebrates, climate change can directly affect fertility rates through induced stress.[20] Factors like the size of large female crabs, spawning conditions (season, geographic range, physical parameters, crab size, and food

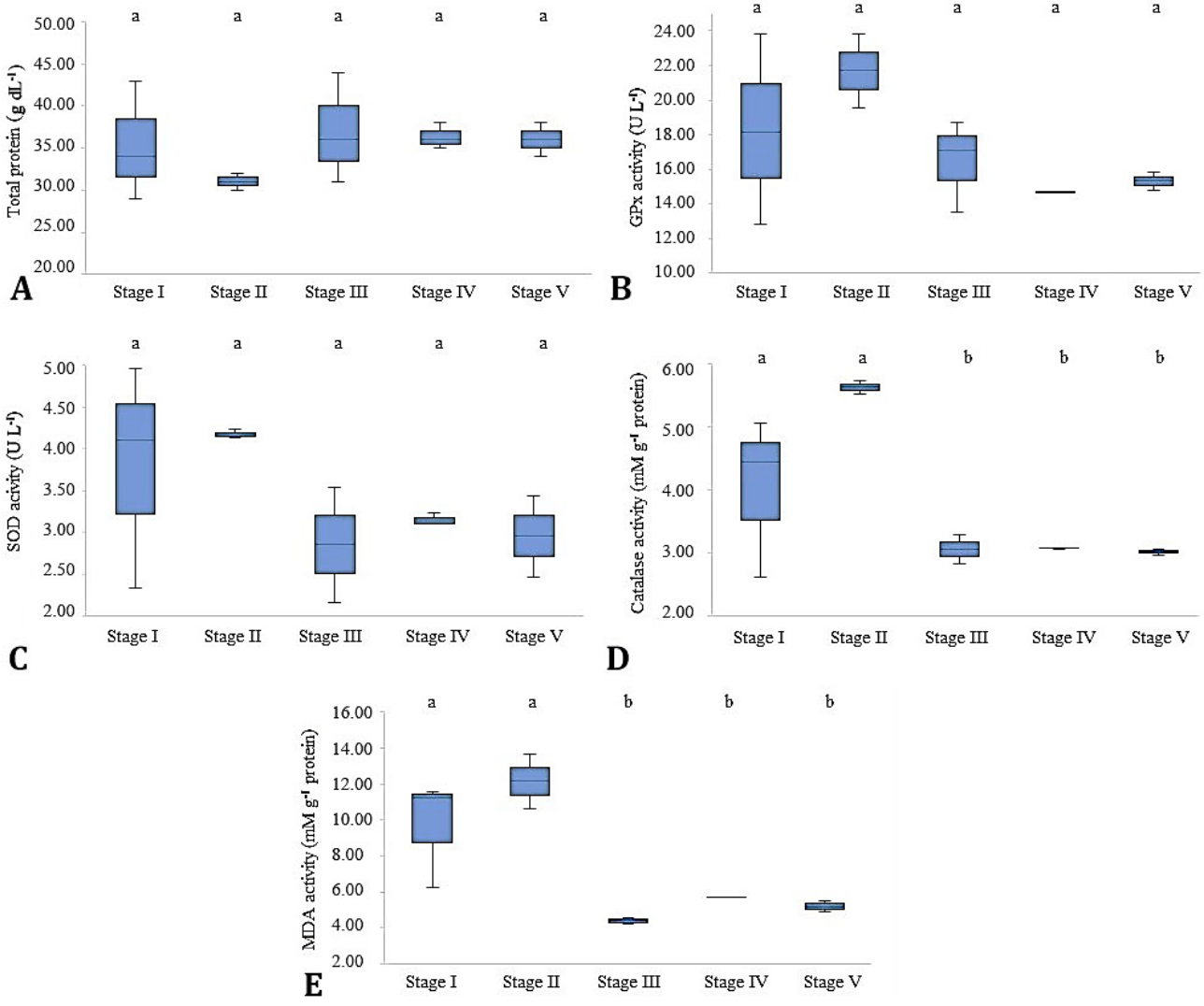

Fig.4. A) Total protein content, **B)** Glutathione peroxidase (GPx), **C)** Superoxide dismutase (SOD), **D)** Catalase, and **E)** Malondialdehyde (MDA); activities of hepatopancreas blue swimmer crab across various the reproductive stages (mean ± SE).
[ab] Statistical differences among the groups are shown with different letters at $p < 0.05$.

availability) impact reproductive success and fecundity in species like *P. sanguinolentus*.[3] The female crab reproductive system comprises H-shaped ovaries near the hepatopancreas. Morphological assessments classify blue swimming crab ovaries into five stages based on size and color consistent with studies on *P. pelagicus*.[1] In *P. segnis*, as indicated by the aforementioned results, the third stage exhibited notable growth with increased carapace size and prevalence of adults. The highest average weight and last abdominal segment measurement occurred in the third and fourth stages indicating full maturity and increased ovarian volume. These findings corresponded with research indicating abundant food in mid-summer and autumn preparing crabs for subsequent stages and spawning. Histologically, the blue swimming crab ovary comprises the ovarian capsule, connective tissue middle layer and germinal epithelium inner layer. The ovarian parenchyma contains numerous follicles at various stages.[1]

The GSI is pivotal for gauging gonad tissue growth and maturation, while the HSI reflects energy storage status. In female crab, GSI typically show a negative correlation due to nutrient transfer from the hepatopancreas to the ovary.[5] The HSI stabilizes during ovarian development with adequate nutrition. In female blue swimming crabs, HSI correlates significantly with sexual maturation stages and ovarian size increase. The peak sexual maturation, nearing spawning, is marked by the highest GSI, notably in the fourth reproductive stage.

The antioxidant system acts as a stress-responsive mechanism, notably influenced by reproductive processes.[3] In aquatic organisms, this system plays a pivotal role in stress mitigation, featuring free radical scavengers and key enzymes like SOD, CAT, GPx, and glutathione S-transferase.[21] These mechanisms counteract the detrimental effects of excessive ROS production.[12] During the reproductive season, oxidative stress manifests with increased protein damage in females.[20] Assessing antioxidant parameters in the blue swimming crab across various stages revealed the development of a complex antioxidant system, safeguarding cell membranes and organelles from ROS during oxidative stress.[22]

During the reproductive period, examination of the crab antioxidant system revealed that SOD, catalase, and GPx reached peak levels in the second ovarian stage. This increase in antioxidant enzymes coincides with the onset of pre-vitellogenesis, signaling biochemical changes induced by this reproductive stage in females.[5] The SOD initiates the conversion of superoxide anions into molecular oxygen and hydrogen peroxide, while elevated catalase and GPx activity indicate subsequent conversion of hydrogen peroxide into water and oxygen, mitigating oxidative stress in liver cells.[18] This study showed significant alterations in SOD and catalase activities in the hepatopancreas tissues indicating their limited capacity to counteract the oxidative effects associated with ovarian changes during sexual maturation.[23] The MDA levels, a marker of oxidative damage, gets peak during the second ovarian stage, which coincides with puberty onset, suggesting a homeostatic mechanism within the antioxidant defense system.[13,24] Elevated ambient temperatures during winter further contribute to oxidative stress, particularly during peak sexual maturity in the fourth and fifth ovarian stages, consistent with findings in *P. trituberculatus*.[9] GPx activity, getting peak during the second ovarian stage, reflects an adaptive response to increased oxidative stress.[7]

Dysregulation of apoptosis, a crucial mechanism for cell death regulation and implicated in diseases like cancer, has been observed in the hepatopancreas of blue swimmer crabs using the TUNEL method.[15] The increase in mitochondrial membrane permeability, a precursor to apoptosis, along with changes in ROS levels and early apoptotic events such as phosphatidylserine externalization, underscores the complex interplay between oxidative stress and apoptosis.[13,25,26] Monitoring these responses is essential for understanding the impact of toxicants and managing the health of crustaceans in polluted environments.[14,27]

The present study investigated oxidative stress effects on protein structures and enzyme activity in blue swimmer crabs. The second ovarian stage showed the lowest total protein levels, suggesting hepatopancreas cells adapt to stress by enhancing enzyme production for detoxification while consuming resources. Fluctuating apoptotic cell levels were observed across reproductive stages, with the second stage displaying the lowest and the first stage of the highest values. Antioxidant system performance varied throughout ovarian development, with the second stage exhibiting the highest GPx activity, indicating heightened oxidative stress response. In early reproductive stages, when gonad development occurs, the antioxidant activity is high, inhibiting apoptosis and minimizing organismal impact. As gonad maturation progresses, it leads to increased protein production and sex hormone activity and less energy is allocated to enzymatic antioxidant production, resulting in increased apoptosis and potentially elevated adult crab mortality.

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Conflict of interests

The authors declare that they have no conflicts of interest.

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