Structure of Hard And Soft Carapace Exoskeleton Biomaterial Through SEM-EDXRS at Various Stages of Development Scylla paramamosain Mud Crab

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Abstract- Crustacean carapace has various functions which can be seen from the composition of the biomaterial in it. Various concentrations of inorganic biomaterial elements were investigated from the hard carapace and the newly molted (soft-shelled) (*Scylla paramamosain*) with SEM-EDXRS (scanning electron microscopy-Energy Dispersive X-ray Spectrometer) technique. This study traced the composition of the inorganic elements of the premolt, postmolt, intermolt and soft (exuvium) crab hard carapace tissue of mangrove crabs from the point of view. Various stages of development. Important elements such as C, O2, Mg, P, Ca, S, Na, Si, Cl, and others, are reabsorbed from the carapace into the body tissues to fulfill further needs in soft-shelled crabs and are reused to some extent during formation new carapace. This study provides evidence that, inorganic elements in freshly molted soft carapace crabs are less common than hard carapace crabs.

Keywords-Carapace, *Scylla paramamosain*, inorganic, SEM-EDXRS

I. Introduction

Mangrove crab, *Scylla* sp. Is in great demand for consumption both in local and export markets because of its deliciousness. The high demand for exports in living conditions and the rising prices of fishery products have made mangrove crab cultivation a part of world interest. Soft carapace crab is a seafood that is highly sought after in international markets such as in Asia, Europe and America which has led to an increase in the production of mud crab which has made the price higher compared to other seafood [1], [2].

In part of their life, mangrove crabs are inhabitants of the high seas, then they immigrate to the brackish water environment of mangrove forests during the post larvae stage [3], [4] and grow quickly mature and mature sex [5]. This species inhabits deep seas as well as brackish waters with muddy or sandy bottoms [6] and classified as a euryhaline species [7] so that it can live in 2-30 ppt salinity waters [8]–[10]. Based on the carapace changing cycle, crustaceans are divided into five stages (A-E), with further subdivisions in each stage. The postmolt period corresponds to stages A1, A2, B1, B2, C1, C2, and C3; the intermolt is the C4 stage; premolt consists of stages Do, D1 ', D1' ', D1' '', D2, D3, and D4; and ecdysis or molting is stage E [11].

Carapace replacement is a biological process in all crustacean species periodically in which the old exoskeleton (exuvium) is released and the new one is formed immediately so that in this process there is an increase in growth or weight [9], [12]–[15]. After the change of carapace, the exoskeleton (exuvium) of crabs becomes smooth or soft due to its soft (spongy) muscles with a thin membrane and accounts for 30-38% of the body weight of freshly molted soft carapace crabs, so it appears that more than 60% of the crab's body is meat [16]–[18]. Towards and when the carapace change takes place, individual crab weight decreases as a result of reduced feeding activity. During the postmolt phase, it will be followed by a relatively constant increase in weight until the next carapace change period [19]–[23]. During the process of carapace replacement, a series of physiological and biochemical changes occur in which some trace elements are absorbed from the old carapace and from the surrounding water through regulation by body tissues and then stored in the gastroliths disk [24] - [26] and then through the excretion process. Reused when forming new carapace and biomineralization of hardening of the carapace [27] - [31].

Crustaceans are known to be able to regulate the total amount of certain chemicals from the appearance of the elements during the metabolic process [32], [33]. In general the variation pattern is well known that Ca, Mg and P during the moulting phase of the calcification process [34], [35]. There is still little information about the elements in the carapace at various stages of the development of *S. paramamosain* mangrove crabs, so research is needed to determine changes in inorganic elements in soft carapace (molting) and hard carapace premolt, postmolt and intermolt in evaluating and providing evidence of inorganic elements. In freshly molted soft carapace crabs are of less variety than hard carapace crabs in the biomineralization process. The benefits of this study will provide information about the bio-regulation of inorganic elements during developmental stages and during the process of replacing old exoskeletons Mangrove crabs from the mangrove forests of Pulokerto Village, Kraton District, Pasuruan Regency, East Java Province

II MATERIAL AND METHOD

Mangrove crabs of *S. paramamosain* were obtained from the mangrove forest area of Pulokerto village, Kraton district, Pasuruan regency, East Java province and then kept in plastic boxes in which there is one each to avoid cannibalism and be able to adjust to normal maintenance pond water. Mud crabs are fed trash fish in the morning and evening purchased directly from local fishermen. Mud crabs are allowed to molt in natural conditions in rearing boxes and are always monitored in the morning, afternoon and evening every day. When you get the crab who had been molted, they were collected on the same day along with the respective old exocuticle and the molts were sacrificed for inorganic analysis. Likewise, crabs that experienced premolt and postmolt from the same environment were collected and stored and then sacrificed after 14 days to compare the concentrations of soft and hard carapace elements. All crabs taken for analysis came from both male and female adult groups with a weight range of 71-90 ± 0.8g and a carapace width of 6.0-7.0 ± 0.7cm. Biometric details of crabs used for analysis are presented in Table 1.

Mud crab samples selected for analysis were grouped separately based on sex, and the stages of its life cycle from premolt, postmot, intermolt to molt (exuvium). Each crab in these groups was then measured for carapace width and body weight. This was done to make it easier to evaluate the presence of their respective elements. The crab samples were then thoroughly washed in deionized water. The premolt, postmolt, intermolt and exuvium phases of hard carapace from freshly molted soft carapace were also taken for analysis. The hard carapace and exuvium samples were dried under the sun and then each was cut into small pieces 0.3 ± 0.2mm using sterile scissors so that they would not be contaminated for further analysis using Scanning Electron Microscope (SEM) - Energy Dispersive X-ray Spectrometer. SEM-EDXS can analyze powerful materials by classifying objects specifically by considering chemical and physical properties and simultaneously providing information on topography, morphology, and elemental composition of objects. Besides, the advantages of being able to detect and analyze surface fractures, provide information in microstructure, examine contamination of a surface, reveal spatial variations in chemical composition, provide qualitative chemical analysis, and identify crystal structure [24] in the calcification process of cuticles in the postmolt and premolt (D3) [36] blue crab, *Callinectes sapidus* and cuticle mineralization processes in terrestrial isopods *Armadillidium vulgare* and *Porcellio scaber* [37], as well as characterization of minerals accumulated in tissues [38].

Instrumentation

Scanning Electron Microscope (SEM) TM 3000 Hitachi with Swift Energy Dispersive 3000 X-Ray Microanalysis. The direct Spectrometer automatically connects to the PC in the circuit by simply plugging it into the computer's USB port. This tool has advantages where the sample to be analyzed does not have to be small in size so that large samples no longer need to be cut and do not require coating as well does not require special specimen preparation like other SEM tools, so the operation is very easy to use to obtain realistic images with good depth of focus and higher magnification as well to determine the composition of the elements.

Hitachi's SEM-EDX-Ray TM 3000 in the elemental specimen analysis process is able to measure the sample density maximum diameter 25mm, magnification from 15 to 30,000 times, has a resolution of 30 nm, 15-kV observation mode and energy-dispersive X-ray spectrometer (EDXS), uses a high-performance SDD detector module and the activation time is only about three minutes on current power (AC) 100–240 V.

III RESULTS AND DISCUSSION

SEM-EDXS managed to find carbon (C), oxygen (O2), magnesium (Mg), and posphorus (P) as the main component of carapace, sodium (Na), chlorine (Cl) and silicon (Si), sulfur (S), bromine (Br), aluminum (Al), potassium (K), iron (Fe), and copper (Cu) in small quantities. Concentrations of 14 constituents of freshly molted exuvium hard and soft carapace in *S. paramamosain* mangrove crabs are presented in Table 2.

Both carbon (C) and oxygen (O2) are the largest elements in all carapace conditions, both hard and soft (exuvium) in both male and female crab sexes. Of the 14 elements found in the carapace, 11 elements are hard carapace in the postmolt stage of male crab, consisting of carbon (C), oxygen (O2),

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magnesium (Mg), phosphorus (P), and calcium sulfur (S), Silicon (Si), clorine (Cl), aluminum (Al), iron (Fe) and copper (Cu). This also occurs in 10 female crab postmolt in the form of carbon (C), oxygen (O2), magnesium (Mg), phosphorus (P), calcium (Ca), sodium (Na), Silicon (Si), clorine (Cl).), bromine (Br), aluminum (Al), and potassium (K). For the second most, there is a premolt stage in both sexes.

The elements carbon (C), oxygen (O2), magnesium (Mg), phosphorus (P), calcium (Ca) and clorine (Cl) are almost all found in hard and soft carapaces (exuvium). These results have also been reported that Ca and Mg are the main elements in the hard and soft carapace of mangrove crabs [39] . The carbon (C) element in both sexes is relatively higher at the time of molting and after passing through it the carbon (C) returns to decline. The highest amount of weight of carbon (C) occurs when crab molting or on soft carapace (exuvium)male crab amounting to 55.32 ± 0.02 wt% and 57.15 ± 0.02 wt% in females compared to the premolt, postmolt or intermolt stages (Figures 1 and 2). The postmolt stage has the second highest carbon element weight (C) under the molting stage in all sexes of 45.38 ± 0.05 wt% in males and 52.86 ± 0.03 wt% in females, while the lowest in both sexes looks the same in the premolt stage of 17.26 ± 0.08 wt% in males and 24.98 ± 0.02 wt% in females. This condition concludes that the element C is absorbed by the soft carapace tissue during the molting process and decreases in number when it enters the next phases such as the premolt, intermolt and premolt phases.

The weight of the element oxygen (O2) contained in the carapace was the highest in the premolt stage and was relatively the same in both sexes, namely 55.01 ± 0.22 wt% in males and 55.51 ± 0.01 wt% in females. Oxygen (O2) tends to decrease in the hard carapace of the postmolt stage in both sexes by 37.29 ± 0.14 wt% in males and 31.51 ± 0.09 wt% in females and increases in the intermolt stage in male or female crab carapace. In the carapace or molting stage, the weight of the element O2 is relatively slightly lower than that of the premolt stage, O2 in the male carapace in all types of carapace from premolt to molting has a relatively higher weight than O2 in female carapace. This element of O2 is thought to always be available in the carapace, the number of which fluctuates depending on the phases in the molting cycle. There have been no studies reported on the element O2 in relation to carapace turnover, so it is difficult to provide a convincing statement regarding its relationship to the carapace turnover cycle in crustaceans.

Magnesium (Mg) is also an element found in all types of hard and soft carapace, both male and female crabs. As a major constituent of exoskeleton crustacean, it is reabsorbed during the premolt period [40]. This element in the two sexes of crabs has a relative weight that is not much different. The highest weight of the element Mg is found in the hard carapace of male and female crabs at the premolt stage. This change in Mg is part of the calcification process. The results of this study are in accordance with the statement [41] that in prawn, the highest proportion of Mg was found in the premolt phase carapace[42], early postmolt white prawn *Penueus indicus*. The weight of Mg in the male hard carapace was higher than that of the female, respectively 1.58 ± 0.60 wt% and 1.48 ± 0.34 wt%, while the lowest in both sexes occurred in the molting (exuvium) stage where Mg in the male exuvium was higher than the female, which was 0.44 ± 0.03 wt% and 0.38 ± 0.07 wt% in females.

The combination of Mg and Asp-rich compounds triggered the basic process of crystallization in the crustacean carapace biomineralization [43]. Crabs have a tendency to produce Mg in calcite as a way of demonstrating strength in finding the mechanical balance of calcite while limiting its solubility in seawater [44],[45] found that Mg levels in *Penaeus californiensis* were stable 12 to 24 hours after molting. [46] states that Mg is required at higher concentrations for proper functioning of the molting process. This suggests that there may also have been chemical or possibly biological (active) deposition of Mg in the old carapace. Such removal of magnesium, even if it happens to be beneficial, because Mg continuously enters the body and has to be excreted [47]. The presence of the element phosphorus (P) is present in all phase in male crabs compared to females. The highest weight of P elements in both sexes is found in exuvium or when the carapace is soft, where the male is 1.45 ± 0.07 wt% and the female is 1.28 ± 0.11 wt%. Especially in the postmolt phase, element P has the lowest weight of all the phases in both sexes, even in females, this element is not found. This element eventually increases in number again in the intermolt phase and then peaks in molting and drops when it is in the postmolt phase. Enhancement inorganic phosphate in the blood when the crab is in the premoult phase can increase the osmotic pressure by 0-2 percent [48].

Calcium (Ca) is seen as the third most abundant element and is present in all stages of the life cycle of crabs, both male and female. The weight of Ca in carapace in male crabs has an average weight greater than that of females. Ca element in premolt in both sexes was seen to have the highest weight of all life phases of crab at 23.60 ± 0.70 wt% in males and 14.30 ± 0.07 wt% in females. During the intermoult period, the exoskeleton the maximally. When crabs undergo carapace change or molting, the weight of Ca in the exuvium of both sexes is seen to be the lowest, namely 2.38 ± 0.33 wt% in males and 2.26 ± 0.05 wt% in females, and then it increases again at postmolt. This change in Ca is part of the calcification process. These data show some evidence that Ca ions and bicarbonate ion reabsorbed from old carapace and temporarily stored in the gastroliths on both sides of the inner belly happened before and after they're actually experiencing replacement of old carapace or molting [49], [50]. This result is in accordance with the statement [51] who reported that Ca was lower in the newly replaced carapace (*Carcinus maenas*) than in the premolt stage.

The crustacean exoskeleton contains a large number of inorganic calcium carbonate compounds, which can reduce flexibility and elasticity so that this hardness interferes with the molting process.

Therefore crustaceans have the ability to reduce the level of CaCO3 content in the cuticles by being reabsorbed and stored before molting to increase flexibility where temporary storage of calcium carbonate is controlled by special organs [52], [53]. The location of Ca storage depends on the crustacean species [54], the terrestrial crustacean *Orchestia cavimana* is stored in a midgut organ called the ceca [55], *Porcellio scaber* and *Armadillidium vulgare* in the sternal area [56], as well as the hepatopancreas or hemolymph [34].

Crustaceans have a high calcium content, which is located in the skeleton because calcium carbonate and Ca are generally cations that are abundant in the body [34]. Ca metabolism is related to life and reproductive strategies [57]. Ca concentrations generally vary in each aquatic ecosystem [40], so that low Ca concentrations can limit growth and production [58]. Therefore, a sufficient amount of elemental Ca is required after each carapace replacement [59]. The imperfection of the calcification process on the soft carapace causes the freshwater crustacean organisms to become cannibalistic [60] and external injuries [61].

Calcium is the main inorganic component present in the carapace and exoskeleton [40], [55], and contains various biological functions (growth and molting) and also has an important structural role in crayfish [62]. The main sources of Ca in crustaceans and crayfish are water and nutrients containing Ca ions [58], [63], [64]. In freshwater crayfish Ca is needed for the new carapace hardening process after molting from water or feed [65]. Calcium (Ca) is also considered as one of the most important minerals [66] and required for normal growth and development of bones and various physiological processes of aquatic organisms [67]. All aquatic organisms require inorganic elements or minerals for their normal life processes [68] and is an important macro element for ecophysiological activity [69], [70].

Water environmental factors such as water temperature, dissolved oxygen, pH, calcium and magnesium play an important role in the growth and survival of crayfish [64], [71], [72], explained that the survival, growth and turnover of the carapace are influenced by calcium, and lobsters that are fed with calcium with added carbonate will have a larger and heavier gastrolith when the carapace is soft because after that calcium carbonate is used for carapace formation rather than being fed in it calcium chloride.

The element chlorine (Cl) is found in all genders but only in the premolt, postmolt and intermolt phases, while in the molting phase in exuvium this element is not found. Like the other eight elements, including sulfur (S), silicon (Si), bromine (Br), aluminum (Al), potassium (K), iron (Fe) and copper (Cu) are found in hard carapaces but are not found in soft carapace (exuvium) of the mangrove crab *S. paramamosain*. The eight elements is thought to have been absorbed from the water that is around it after the replacement of the old carapace which is regulated in the body's tissues during the excretion process or is used during the process of forming new carapaces and also indicates that these elements may have been excreted during the replacement of old carapace.

Br is an element that also accumulates in crustaceans [73], which consists of non-calcified Br-rich tissue containing about 1% Br. This Br showed higher resistance to fracture and hardness compared to calcified cuticles and was found to bind to the phenyl ring to form bromotyrosine which attributed increased hardness and stiffness to the promotion of Br-induced protein crosslinking and / or when a higher Br mass [74]. Cu elements in this study were only found in the postmolt and premolt stages of crab hard carapace in both sexes. This result differs from research [75], [76] [75] which states that Cu is present in the intermolt phase and in the hemolymph and hepatopancreas [77].

In this study also found elements of Al, K, and Fe which were only found in the postmolt and premolt stages of crab hard carapace in both sexes. It is possible that these elements are arranged after the carapace change process occurs and there has been no previous research that has reported these elements from crustaceans in relation to carapace turnover, so it is difficult to provide a convincing statement of their relationship to the carapace change cycle.

IV Conclusion

The study shows the technique's effectiveness in analyzing the biological material of carapace tissue samples, thus opening up opportunities for further broader studies.. Inorganic biomaterials that are present especially in the molting phase or when the crab changes to a new carapace with a soft carapace (exuvium), both male and female have biomaterial elements that are relatively different from all types of hard carapace at the premolt, postmolt and intermolt stages. Comparing the elements in the hard and soft carapace (exuvium) of male and female *S. paramamosain* crabs (Figures 1 and 2) shows that Mg in the premolt stage to molt is the second-highest concentration after Ca, and then both drastically decrease when the molt stage. Towards postmolt. It is clear that Ca, Mg, and other constituents are also partially reabsorbed [78], [79] from the hard (old) carapace into the tissues of the body as a necessity when the molting process takes place and is further used for the formation. As well as new carapace hardening [50], [80], [81] to some extent [82] - [84] in the biomineralization process [34]. This condition can be concluded clearly that the presence of elemental concentrations in the carapace will be affected when the crab enters the new carapace replacement stage (molt).

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Table 1. Biometric data of *S. paramamosain* mud crab samples.

S. paramamosain	Life stages	Carapace width (CW) (cm)	Weight (BW) (g)
Male			
hard carapace	PreMolt	7.47 ± 0.15	79-90 ± 13.2
	PostMolt	8.57 ± 0.23	100-110 ± 8.0
	InterMolt	7.73 ± 0.45	81-87 ± 10.4
soft carapace (exuvium)	Molt	7.77 ± 0.35	98-106 ± 2.5
Female			
Carapace hard	PreMolt	7.13 ± 0.29	76-80 ± 5.5
	PostMolt	8.57 ± 0.23	93-100 ± 3.7
	InterMolt	7.27 ± 0.50	75-80 ± 7.1
Soft carapace (exuvium)	Molt	8.2 0 ± 0.52	90-99 ± 5.4

Table 2. Percentage by weight of various elements of *S. paramamosain* crab carapace (in wt%) using the SEM-EDXS technique.

Element	Male crab (n = 12)				Female crab (n = 12)			
	Premolt	Postmolt	Intermolt	Molt	Premolt	Postmolt	Intermolt	Molt
	Carapace hard			Exuvium	Carapace hard			Exuvium
	(wt%)							
Carbon (C)	17.26 ± 0.08	45.38 ± 0.05	21.06 ± 0.16	55.32 ± 0.02	24.98 ± 0.02	52.86 ± 0.03	39.38 ± 0.14	57.15 ± 0.02
Oxygen (O2)	55.01 ± 0.22	37.29 ± 0.14	54.22 ± 0.32	40.40 ± 0.54	55.51 ± 0.01	31.51 ± 0.09	43.96 ± 0.01	38.91 ± 0.56
Magnesium (Mg)	1.58 ± 0.60	0.72 ± 0.76	1.49 ± 0.08	0.44 ± 0.03	1.48 ± 0.34	0.83 ± 0.16	0.88 ± 0.16	0.38 ± 0.07
Phosphorus (P)	1.20 ± 0.24	0.00	0.46 ± 0.09	1.45 ± 0.07	0.84 ± 0.05	-	0.65 ± 0.04	1.28 ± 0.11
Calcium (Ca)	23.60 ± 0.70	8.17 ± 0.01	18.47 ± 0.26	2.38 ± 0.33	14.30 ± 0.07	4.90 ± 0.06	13.63 ± 0.28	2.26 ± 0.05
Sulfur (S)	-	0.33 ± 0.13	-	-	-	-	-	-
Sodium (Na)	0.71 ± 0.08	-	1.34 ± 0.01	-	1.22 ± 0.21	0.51 ± 0.02	0.69 ± 0.44	-
Silicon (Si)	-	3.12 ± 0.63	-	-	0.29 ± 0.05	0.57 ± 0.08	-	-
Chlorine (Cl)	0.60 ± 0.15	0.63 ± 0.03	0.92 ± 0.08	-	1.10 ± 0.03	0.97 ± 0.07	0.78 ± 0.32	-
Bromine (Br)	-	-	-	-	-	3.40 ± 0.58	-	-
Aluminum (Al)	-	2.64 ± 0.02	-	-	-	1.32 ± 0.04	-	-
Potassium (K)	-	-	-	-	0.26 ± 0.17	0.47 ± 0.26	-	-
Iron (Fe)	-	1.69 ± 0.25	-	-	-	-	-	-
Copper (Cu)	-	0.00	-	-	-	-	-	-

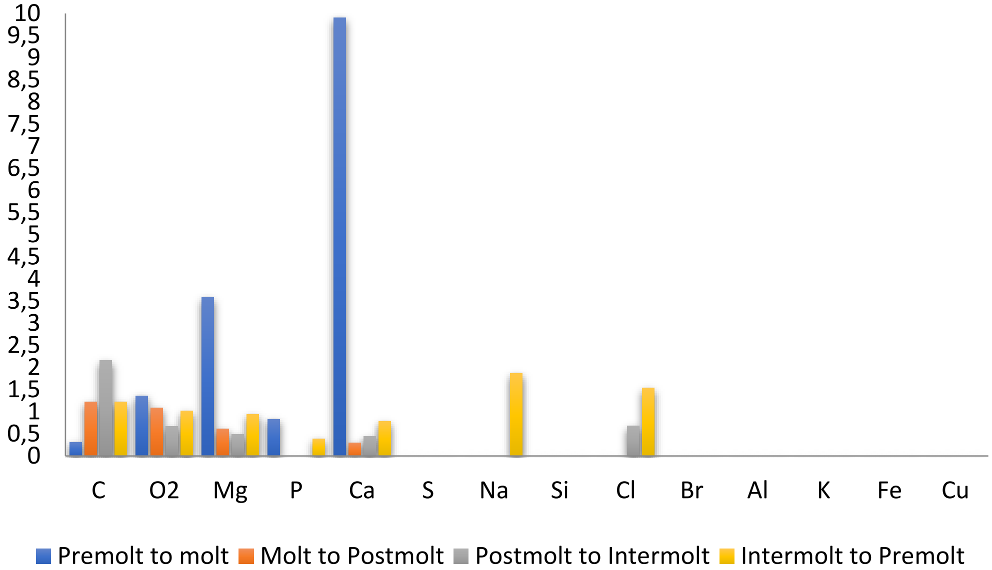

Figure 1. Comparison of the elements in hard and soft carapace (exuvium) of male crab *S. paramamosain*

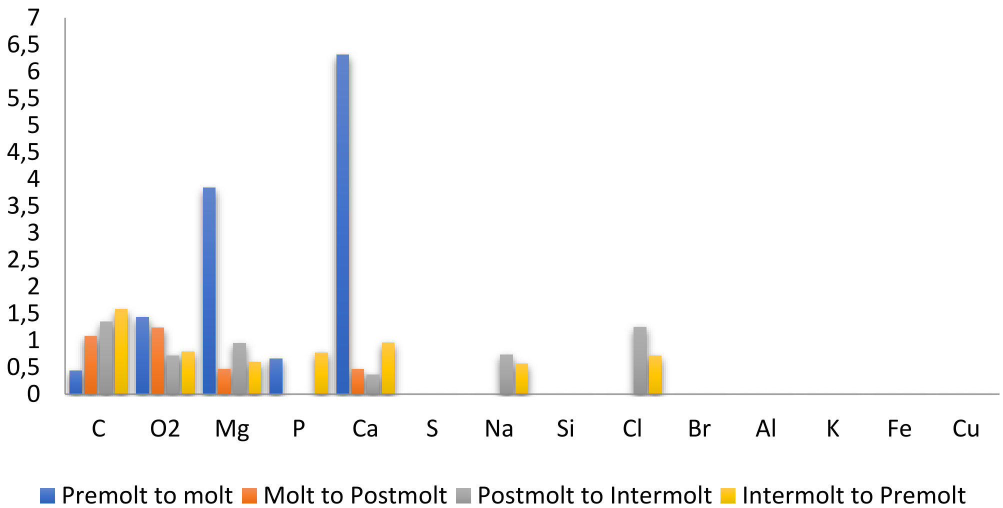

Figure 2. Comparison of the elements in the hard and soft carapace (exuvium) of female *S. paramamosain* crabs