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Natural and Induced (Eyestalk Ablation) Molt Cycle in Freshwater Rice Field Crab *Oziothelphusa Senex Senex*

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Abstract

In crustaceans, molting is the process of shedding old exoskeleton and is required for the somatic growth of new exoskeleton for its growth. The molting processes in crustaceans vary from one species to other and also vary with the environmental conditions. In the present study the molt cycle of freshwater crab Oziothelphusa senex senex was studied. The size of crabs selected for the study is 30 ± 2 g. The natural molt cycle is consisting of intermolt (C1, C2, C3 and C4), premolt (D1, D2, D3 and D4), ecdysis (E) and post molt (A1, A2, B1 and B2) stages were measured and the percentage of each stage in the molt cycle was calculated. The biggest stage in the O. senex senex molt cycle is intermolt stage (90.0%) and shortest one is ecdysis (0.01%). Induced molt cycle was studied by eyestalk extirpation (ESX) and observed that 60.71% of male crabs and 52.0% female crabs were molted after 28th day of extirpation suggesting the role of eyestalk principle in regulating the molting process.

Keywords: Molt cycle; Eyestalk ablation; Maxillipede; Setae; *Oziothelphusa senex senex*

Introduction

Arthropods, encased in protective cuticles have solved the problem of size increase by a discontinuous growth pattern, the molting cycle. Molting is one of the most important physiological processes occurring during the arthropod life cycle and is controlled by molt-inhibiting hormone (MIH) and molting hormone, ecdysone [1-3]. In crustaceans, the growth cycle consists of a series of molts following intermolt periods. The growth in a typical decapod crustacean is a step wise process happening during the molt, while the resources for growth are gathered during intermolt period [4]. The growth, weight gain takes place during the molt itself, when the animal takes water into the body and thus increases in size, weight and volume. In crabs, the weight gain at molt is normally between twenty and thirty percent of premolt stage weight. During intermolt the animal substitutes its water content with tissue growth, and the weight gain during the long intermolt is normally less than 5% of immediate postmolt weight. Also, the water content of hemolymph decreases during the intermolt [5].

In crustaceans, the conventional method for inducing growth is eyestalk ablation (ESX) that involves the removal of molt inhibiting hormone (MIH) and release of molting hormone the ecdysone. In which MIH is produced in the eyestalk and molting hormone is produced in Y-organ. By eyestalk ablation MIH action is excluded or suppressed simultaneously it allows molting hormone to act. Thus on removal of eyestalk causes increase of ecdysteroid secretion from Y-organ which includes precocious molting in many decapods species [6-9]. On removal of eyestalk, mortality increases because it removes four ganglia from each eyestalk, a considerable portion of central nervous system thereby induces hormonal imbalance and stress [10]. Besides this decreased hemolymph osmolarity by ESX has been reported in crustaceans [11,12] Along with growth unilateral ESX also shortens molt interval and stimulate gonad maturation is evidenced in several crustaceans like *Metapenaeus dobsoni* [12] *Astacus leptodactylus* [13] *scylla serrata* [8].

The criteria used in the present study to characterize the molt cycle stages in the crab *O. senex senex* is essentially similar to the stage description developed by Drach [14,15] and other workers (see the review of) Kurup [16] and redescribed by Reddy [17]. Though, the change in behavior and morphology at the stages of the molt cycle of *O. senex senex* broadly similar to those of other decapod crustaceans, the duration of the various stages and the characteristics used in their recognition vary widely. However no clear demarcation of the molt stages of natural molt cycle has been noted in the crab *O. senex senex* and hence the present study is aimed to focus on the measurement of each and every sub-stage of natural molt cycle along with role of eyestalk on molting in crab *Oziothelphusa senex senex*.

Materials and Methods

Animals

The freshwater rice field crab, *O. senex senex* (30 ± 2g) were used as experimental animal for the present investigation. The crabs (both male and female) were collected from rice field and irrigation canals (free from pesticides and pollutants) in and around Renigunta (13.6°N and 79.5°E) Andhra Pradesh (South India). The crabs were brought to the laboratory and maintained in the laboratory at 28 ± 1°C in tubs partially filled with aged fresh water. They were acclimatized to laboratory conditions (12:12 L:D) for at least 7 days before being used in experiments. The water in the tubs was changed daily. During the experiment, the crabs were fed on sheep meat ad libitum. Feeding was stopped one day before the commencement of experiment to avoid any internal complications.

Determination of natural molt cycle

A total of 350 animals (200 males and 150 females) were used to

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study the natural molt cycle in this study. The natural molt cycle stages in the freshwater crab *Oziothelphusa senex senex* were determined according to the method described by Drach [14,15], modified by Reddy [17]. The molt cycle in the crab *O. senex senex* consists of four molt periods viz., postmolt, intermolt, premolt and ecdysis. The postmolt period (divided into two stages A and B) is sub-divided into four stages, viz., A_1, A_2, B_1 and B_2; the intermolt into C_1, C_2, C_3 and C_4 and the premolt into D_0, D_1, D_2, D_3 and D_4. The duration of total molt cycle of the crab *O. senex senex* observed by setal development in the mastigobranch of third maxillipede and the characteristic features of molt cycle were summarized. The experiments were conducted twice during the months of June and July in 2014 and 2015.

Role of Eyestalk ablation on molting

In the present study, in order to determine the role of eyestalks in the regulation of molting in the crab *O. senex senex*, bilateral eyestalk ablation experiments were conducted in both males and females. The major purpose of eyestalk ablation is to induce molting activity in the crab under laboratory conditions.

Experiments for male and female crabs were conducted individually. A total of 130 male and 130 female crabs with body weight of 28 ± 2g were divided into three groups for each gender. For each experiment animals were divided into three groups. Group I served as controls with 10 animals and sacrificed on day '0' of experiment and recorded the molt stage. Group II served as concurrent control and were maintained along with experimental animals. Group III served as ESX and maintained up to 28 days. Selected number (Tables 1 and 2) of group II and III animals were sacrificed on 7th, 14th, 21st and 28th day of experiment to determine the molt stages. The molt stages were determined by observing the setal development in the mastigobranch of third maxillepede.

Results and Discussion

Measurement of postmolt stage

The postmolt stage starts immediately after molt and it occupies 5.3% of total molt cycle duration. The characteristic feature of postmolt is that of hardening and calcification of the carapace. Postmolt was divided into stages A and B, and are sub-divided into A_1, A_2, B_1 and B_2 respectively.

Stage A: The duration of stage A is 2.7% of total molt cycle duration and 50.94% (Figure 1) of postmolt duration. Stage A begins immediately after a crab has cast the exuvium. The stage is dominated by absorption of water, when a crab expands its volume up to 50%. Due to water absorption, the weight of the body is fluctuating.

In sub-stage A_1 the animal is extremely quiescent and the exoskeleton was as soft as, parachment and shiny. The body appears dark brown or black in colour. The size of the body increased in freshly molted animal due to rapid water absorption and the animal appears blotted. At this sub-stage, the legs of the animal are not functional and the crab cannot lift its body on its legs or support its weight. The crab does not feed at this sub-stage and it occupies 0.9% of total molt cycle duration (Table 3) and 16.98% of postmolt duration (Figure 1).

In sub-stage A_2, the crab is still inactive and is unable to lift its body on its legs, but moves with difficulty. At the end of this sub-stage the expansion of exoskeleton is completed and its colour becomes shiny black. The sub-stage A_2 occupies 1.8% of total molt cycle duration (Table 1) and 33.86% of postmolt duration (Figure 1). The animal is inactive and non-feeding at this stage.

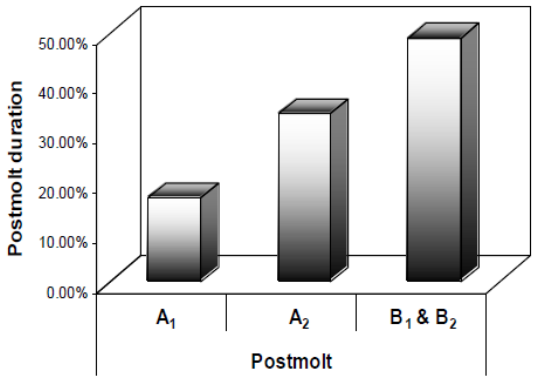

Figure 1: The duration of different sub-stages of post molt in the crab *Oziothelphusa senex senex*.

Group		Days after eyestalk ablation				
	Day '0'	1	7	14	21	28
Control (n=10)	C_4 (10.0)	-	-	-	-	-
Concurrent control (n=20)	-	C_4 (4.0)	C_4 (4.0)	C_4 (4.0)	C_4 (4.0)	C_4 (4.0)
Eyestalk Ablated (n=100)	-	C_4 (8.0)	C_4 (6.0)	D_1 (10.0)	D_1 (3.0)	D_2 (2.0)
			D_1 (2.0)	D_2 (4.0)	D_3 (4.0)	D_4 (4.0)
				D_3 (4.0)	D_4 (5.0)	D_4 (5.0)
					Molted (10.0)	Molted (17.0)
Values in parentheses are number of animals.						
16 crabs died after eyestalk ablation during experimentation.						

Table 1: Different molt stages of the male crab on different days after eyestalk ablation.

Stage B: In stage B, the hardening of exoskeleton takes place. Feeding does not yet begin. Stage B occupies 2.6% of total molt cycle duration (Table 1) and 49.16% of postmolt duration (Figure 1). In the sub-stage B_1, the prothoracic area becomes hard and the other areas of carapace (mesogastric, urogastric, cardiac, anterior and posterior bronchial regions) become firm but easily depressible. Propodus and merus of chelate leg are hard but can be bent without cracking. At the end of the sub-stage B_1 crab starts feeding. In sub-stage B_2, only mesogastric and urogastric areas attain rigidity but the anterior and posterior branchial regions are in firm state. In this sub-stage, carpus and propodus are assum uniform hardness.

Measurment of intermolt or stage C

Intermolt or stage C is the longest period (occupying 90.0%) of the total molt cycle (Table 1), in which the animal leads a normal life. The water content of the crab is gradually replaced by tissue. During this stage the exoskeleton is mineralized and it assumes the typical rigid form. The sub-stage C_1 occupies 3.6% of total molt cycle duration (Table 1) and 4.0% of intermolt duration (Figure 2). Except cardiac area of carapace, the remaining regions are hardened and the body has achieved its definite colouration. In sub-stage C_2 the entire carapace regions are hard. The appendages of crab are also uniformly hard and the animal feeds actively in this sub-stage. The sub-stage C_2 occupies 6.4% of total molt cycle duration (Table 1) and 7.11% of intermolt duration (Figure 2).

In sub-stages C_3 and C_4 the animals show normal locomotor and prandial activity. These two sub-stages cannot be strictly demarcated in

the present crab. The duration of these sub-stages lasts few months and is depends on internal and external conditions. In case of laboratory maintained animals, the duration of these sub-stages is more than the natural ones. However, in the total molt cycle, the duration of these sub-stages occupy nearly 80.00% (Table 1) and 88.89% of intermolt duration (Figure 2). In these stages, the crabs show normal locomotor and feeding activity.

Measurment of premolt or stage D

Stage D is the premolt period of molt cycle and it occupies 4.69% of molt cycle duration (Table 1). During this stage, the crab prepares for the molt by accumulating reserves, reabsorbing the calcium from the shell and synthesizing the new layer of new cuticle. The crab ceases feeding at some point of time during this stage. The characteristic feature of premolt is formation of new setae in the mastigobranch of third maxillepede. This phenomenon was first observed by Braun [18] and is of great importance in determining the incipience of molting. In *Oziothelphusa senex senex*, the mastigobranch of third maxillepede at intermolt (sub-stage C_4) contain numerous hair-like setae (Figure 3).

In the sub-stage D_0, the underlying epidermis was separated from the exoskeleton and it is termed as apolysis. In sub-stage D_1 the retraction of epidermal layer from old cuticle and loss of external setae was observed with appearance of rudiments of setal grooves from the old epidermal layer in mastigobranch for development of new setae (Figure 4). These sub-stages (D_0 and D_1) occupy 0.3% of the total molt cycle duration (Table 1) and 6.39% of premolt duration (Figure 5).

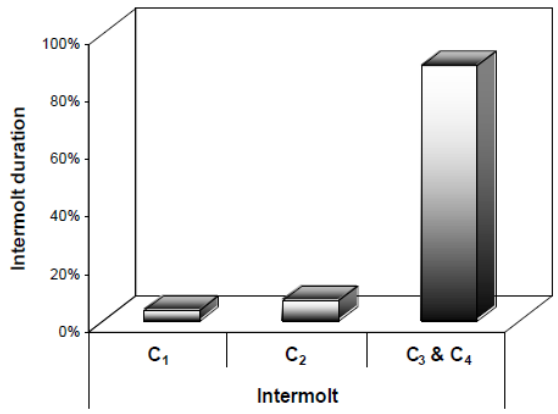

Figure 2: The duration of different sub-stages of intermolt in the crab *Oziothelphusa senex senex.*

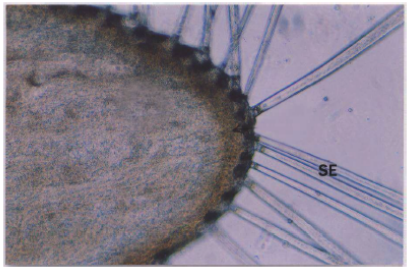

Figure 3: The intermolt (stage C4) mastigobranch of third maxillepede of *Oziothelphusa senex senex* showing external setae (SE) 100 X.

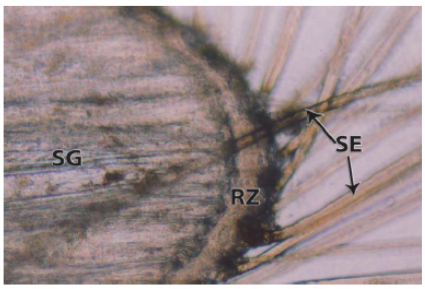

Figure 4: The mastigobranch of third maxillepede of premolt (sub-stage D2) crab showing newly formed setal grooves (SG) with retraction zone with external setae (SE). 100 X.

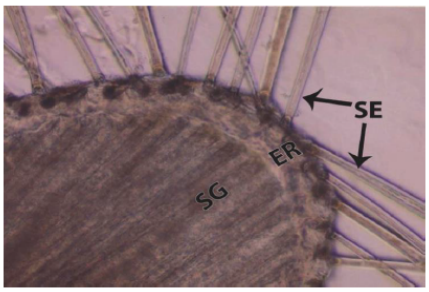

Figure 5: The mastigobranch of third maxillepede of premolt (sub-stage D1) crab showing retraction of epidermal layer.

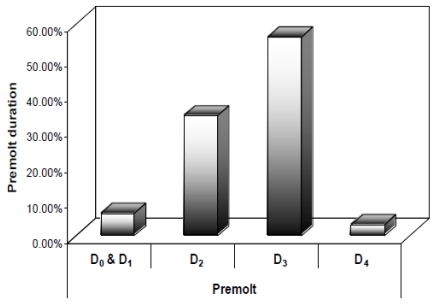

Figure 6: The duration of different sub stages of premolt in the crab *Oziothelphusa senex senex.*

The sub-stage D_2 occupies 1.6% of the total molt cycle duration (Table 1) and 34.08% of premolt duration (Figure 6). In this sub-stage, the new pigment layer is synthesized. This layer is underneath of old exoskeleton and can be observed only by removing a part of old exoskeleton. The newly formed setal grooves in the mastigobranch are clearly visible and easily defined (Figure 4).

In sub-stage D_3 the crab ceases feeding and locomotion. The newly formed integument has become completely detached from the old exoskeleton. A remarkable colour differentiation was observed between old exoskeleton and newly formed integument. The setal articulation and the newly formed setae are clearly visible and discretely arranged within the setal grooves. The retraction zone formed in between the epidermal layer and cuticle was more and clearly demarcated (Figure 7). In this sub-stage, due to the re-absorption of calcium from old exoskeleton, the hemolymph of the crab is milky. The sub-stage D_3

occupies 2.64% of the total molt cycle duration (Table 1) and 56.34% of premolt duration (Figure 6).

The sub-stage D_4 occupies only 0.15% of the total molt cycle duration (Table 1) and 3.19% of premolt duration (Figure 6). In sub-stage D_4, the line of dehiscence is well marked and is characterized by the opening of the line. The inner newly formed integument is dark brown or black in colour and remains detached from old exoskeleton. The carapace becomes very thin and papery in texture and breaks easily under slight pressure. At this sub-stage due to the movement of new setae to the peripheral region of mastigobranch, the disappearance of epidermal retraction zone was observed in the base of the mastigobranch (Figures 8a and 8b). The hemolymph of the animal is milky and the crabs are inactive and cease feeding.

Measurment of Ecdysis or Stage E

Stage E, or ecdysis is shortest period in the molt cycle and it occupies only 0.01% of the total molt cycle duration (Table 1). Ecdysis is the shedding of exoskeleton and not considered as a part of classical molt stages. All the changes observed in the premolt period lead to shedding of the old exoskeleton. During stage E nearly all the activities of the crab ceased. Active withdrawal of the old exoskeleton lasted slightly more than 15 minutes in five instances observed in full; but if withdrawal prolonged abnormally, it usually ends in death. All the appendages like legs and mouth parts are withdrawn from the old exoskeleton during exuviation. During exuviation the crab is completely helpless. All the crabs are molted late at night except one. When ecdysis is completed, the animal becomes quiescent again, but pumps water through its mouth at the maximal rate. The exoskeleton of freshly molted crab becomes soft and is stretched due to absorption of water. The size of the animal after ecdysis found to be much larger than the old one.

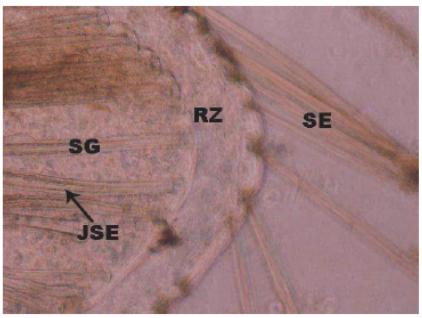

Figure 7: The mastigobranch of third maxillepede (sub-stage D3) of the crab *Oziothelphusa senex senex* showing setal articulation (SA), newly formed juvenile setae (JSE) in the setal grooves (SG) and clear retraction zone (RZ) in between epidermal layer and cuticle. SE = external setae; 100X.

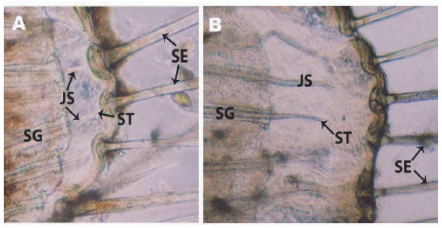

Figure 8: The mastigobranch of third maxillepede showing early sub-stage D4 (A) and late sub-stage D4 (B) crab showing the extruviation of juvenile setae (JS) from the setal grooves (SG) with distinct setal tips (ST). SE = external setae; 100X.

Role of eyestalks on molting

The experimental animals were observed to determine the molt stages on first, seventh, fourteenth, twenty first and twenty eighth day after eyestalk ablation. The molt stages were determined by observing the setal development in the mastigobranch of third maxillepede. The incipience of premolt stage was observed 7 days after eyestalk ablation in males, but the percentage of males entered into the premolt is less (25.0) (Table 2). In females, the premolt substages D_1 and D_2 were observed 14 days post eyestalk extirpation (Table 3), indicating the molt initiation was started after the day 7 and before day 14 after eyestalk ablation. The males exhibited D_1, D_2 and D_3 sub-stages of premolt on the 14th day of experiment (Table 2). On the 21st day after eyestalk ablation some of the males and females exhibited advanced premolt stages (stage D_3 and D_4) and some are molted (Tables 2 and 3). The percentage of males and females entered into different sub-stages of premolt and molted on the 21st day after eyestalk ablation was presented in Figure 9. On this day of experiment percentage of molted females (34.78) is lesser than the molted males (45.45). Most of the eyestalk ablated males and females exhibited advanced premolt sub-stages (stage D_3 and D_4) and several crabs molted on the 28th day of experiment. Figure 10 shows the percentage of eyestalk ablated males and females entered in to the different sub-stages of premolt and molted on 28th day after eyestalk ablation. On this day of experiment, the percentage of males (60.71) molted is higher than the females (52.0). None of the crabs in concurrent control groups (both the sexes) entered into premolt even after 28 days (Tables 2 and 3), indicating that no other factor involved during the experimentation on molting. The percentage of mortality during experimental period in an eyestalk ablated males and females were 16.0 and 20.0 respectively.

Group		Days after eyestalk ablation				
	Day '0'	1	7	14	21	28
Control (n=10)	C4 (10.0)	-	-	-	-	-
Concurrent control (n=20)	-	C4 (4.0)	C4 (4.0)	C4 (4.0)	C4 (4.0)	C4 (4.0)
Eyestalk Ablated (n=100)	-	C4 (8.0)	C4 (8.0)	D1 (10.0)	D1 (5.0)	D2 (3.0)
				D2 (6.0)	D3 (4.0)	D4 (4.0)
					D4 (5.0)	D4 (4.0)
					Molted (7.0)	Molted (14.0)
Values in parentheses are number of animals.						
20 crabs died after eyestalk ablation during experimentation.						

Table 2: Different molt stages of the female crab on different days after eyestalk ablation.

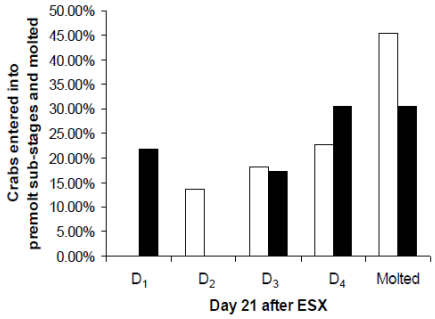

Figure 9: The cumulative percentage of crabs (male and female) entered into premolt and molted on 21st day after eyestalk ablation (ESX). Open bars - Males; solid to bars – females.

Molt stage	Sub- stage	Feeding	Activity	Exoskeleton	Epidermis	Diagnostic characters	% of total molt cycle
Post molt							**5.3**
A	A1	None	Weak	Soft and shiny	Transparent	Epidermis closely applied to new cuticle tip; internal tissues disorganized.	
	A2	None	Weak	Soft	Granular	Epidermis closely applied to new cuticle tip; internal tissues disorganized.	2.7
B	B1	None	Restored	Hardening	Granular	Chromatophores visible and internal tissues appear	2.6
	B2	Minimal	Minimal	Hardening	Granular	Appearance of more chromatophores.	
Intermolt							**90**
C	C1	Minimal	Maximal	Hard	Granular	Internal cone formation begins; setal organs become visible.	3.6
	C2	Maximal	Maximal	Hard	Granular	Completion of internal cone formation.	6.4
	C3	Maximal	Maximal	Hard	Granular dense	Granular matrix retraction completed.	80
	C4	Maximal	Maximal	Hard	Granular and very dense	The setal lumen was pinched off at the base of the setae near the setal articulation.	
Premolt							**4.69**
D	D0	Maximal	Maximal	No new cuticle yet	Apolysis (retraction)	No setal development was observed.	0.3
	D1	Minimal	Maximal	Appearance of new pigmented layer.	Invaginates	Initiation of setal articuloation.	
	D2	Minimal	Maximal	Gap formation between old and new cuticle	Invaginates	Increase of setal articulation.	1.6
	D3	Stopped	Weak	Thinning of old cuticle	Invagenates	Development of new setae and milky hemolymph.	2.64
	D4	None	Ceased	Paper-like old cuticle	Completion of invagintion	Withdrawal of old setae & formation of new setae.	0.15
Ecdysis	E	None	Ceased	Old cuticle is shed	Transparent	Soft body	**0.01**

Table 3: The characteristic features of molt cycle in the fresh water rice field crab *Oziothelphusa senex senex*.

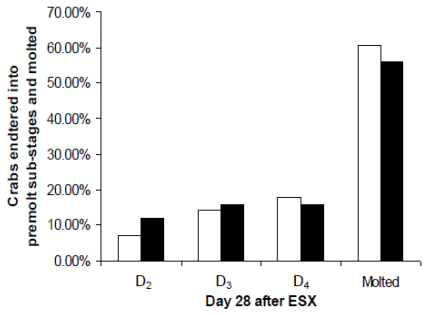

Figure 10: The cumulative percentage of crabs (male and female) entered into premolt and molted on 28th day after eyestalk ablation (ESX). Open bars - males; Solid bars – females.

The role of eyestalks in the regulation of molting was studied in several crustaceans see reviews [19-21] have also observed the shortened molt cycles in the eyestalk ablated male and female prawns *Macrobrachium rosenebergii*. Precocious molting has been well documented in spider crab *Libinia emarginata* [22] and freshwater prawn *Macrobrachium rosenbergii* after eyestalk ablation [23]. Though eyestalk ablation induced precocious molting in several species of crustaceans, the same technique failed to induce molting in spiny lobster *Panulirus argus* [24]. In the same species Travis [25] reported that eyestalk ablation does accelerates precocious molting as well as gonadal activity but not simultaneously, indicating the molting and reproduction in this lobster are antagonistic events.

The X-organ sinus gland complex is located in the eyestalks and is responsible for the synthesis, storage and release of different peptide hormones. Molt-inhibiting hormone (MIH) is one of the important eyestalk peptide hormones and is involved in the regulation of molting. MIH regulates the molting process by suppressing the synthesis and release of molting hormone, ecdysone, by Y-organs (molting glands). Freeman and Bartall [26], and Bruce and Chang [27] have demonstrated the presence of molt-inhibiting hormone in the eyestalks of *Penaeus pugio* and *Homarus americanus* respectively. Ablation of eyestalk leads to elimination of MIH ultimately the molt induction [23]. The decreased levels of MIH in the hemolymph was observed after eyestalk ablation [28] and at the same time the increased ecdysteroid levels were also observed in the hemolymph [29,30]. Decreased titers of hemolymph ecdysteroids during the intermolt, maximum levels during the premolt and low levels just prior to ecdysis was observed in crayfishes *Orconectes sanborni* [31] and *Procambarus clarkii* [32] and in the blue crab *Callinectes sapidus* [33].

In the last decade the level of MIH mRNA expression in the X-organ of the blue crab *Callinectes sapidus* [34] and the MIH content in the sinus glands of the crayfish *Procambarus clarkii* [32] and *Cherax quadricarinatus* [3] were estimated at each stage of molt cycle. Nakatsuji and Sonobe [35] demonstrated the changes in the hemolymph MIH levels during the molt cycle in the American crayfish *Procambarus clarkii*. They observed the titer of hemolymph MIH at intermolt stage (6.53 fmol/ml) was about five times higher than that at the early premolt stage (1.28 fmol/ml). The above studies clearly indicates the role of MIH in the regulation of molting in crustaceans.

The another eyestalk neuropeptide called mandibular organ-inhibiting hormone (MOIH) also involved in the regulation of molting in crustaceans. MOIH inhibits the synthesis and secretion of sesquiterpenoid hormone methyl farnesoate (MF) by mandibular organs. MF is known to regulate molting in many crustacean species [36-38].

The possible involvement of mandibular organ in the regulation of molt cycle was demonstrated in several crustaceans. Nagaraju et

al., [36] observed the size of mandibular organs at different stages of molt cycle in the crab *O. senex senex* and reported an increase in the size of the mandibular organ when the crab enters in to premolt. The morphological changes of the mandibular organ during the molt cycle in the American lobster *Homarus americanus was* also studied [39]. Hinsch [40] observed the increased synthetic activity of the mandibular organ during induced molt cycle along with the ultra-structural changes in the male spider crab, *Libinia emarginata*. The increased synthetic activity of the mandibular organ during premolt period may be due to the release of mandibular organ cells from suppression of methyl farnesoate synthesis by MOIH. It is clear that the eyestalk peptide hormones inhibit the synthesis and secretion of MF from mandibular organs and ultimately molting.

In the present study, the surgical extirpation of the eyestalk resulted in shortened molt cycle intervals in the crab *O. senex senex*. This may be due to the reduction of titers of MIH, and/or MOIH from circulation after the removal of eyestalks thereby results in increased levels of MF and ecdysteroids from mandibular organ and Y-organ respectively. The increased levels of MF and ecdysteroids are known to shorten the molt cycle duration.

The natural molt cycle with demarcated molt stages have elucidated in the present study in the crab *O. senex senex*. The detailed natural molt cycle of crab may show a way in studying induced molt cycle with various molecules/factors like xenoestrogens, various metals, vertebrate type steroids, biotic and abiotic factors etc., including manipulation of crustacean hormones. Eyestalk extirpation induced molting with reduced molt cycle duration but found mortality suggesting the need for search an effective alternative method for induced molt in crustaceans.

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