Zoological Studies **51**(7): 966-976 (2012)

Zoological Studies

Hemolymph Ecdysteroid Titers in a Brachyuran Crab *Uca triangularis* that Concomitantly Undergoes Molting and Reproduction

Sudha Kappalli[1], Nagathinkal T. Supriya[1], Velayudhan Krishnakumar[1], Anilkumar Gopinathan[2,*], and Ernest S. Chang[3]

[1]*Post Graduate Department of Zoology and Research Centre, Sree Narayana College, Kannur 670007, Kerala, India*
[2]*School of Biosciences and Technology, VIT University, Vellore 632014, TN, India*
[3]*Bodega Marine Laboratory, University of California-Davis, Bodega Bay, CA 94923, USA*

(Accepted May 11, 2012)

Sudha Kappalli, Nagathil T. Supriya, Velayudhan Krishnakumar, Anilkumar Gopinathan, and Ernest S. Chang (2012) Hemolymph ecdysteroid titers in a brachyuran crab *Uca triangularis* that concomitantly undergoes molting and reproduction. *Zoological Studies* **51**(7): 966-976. Investigations conducted thus far suggest that premolt growth and ovarian growth are antagonistic events in brachyuran crabs, arguably implying that yolk deposition does not occur during premolt when the ecdysteroid titer is very high. The present paper examines ecdysteroid levels in a brachyuran crab *Uca triangularis* that exhibits simultaneous programming of premolt growth and reproduction during certain seasons of the year. The species exhibits 2 patterns of breeding cycles, 1 (premolt breeding cycle) synchronous with the premolt cycle during Feb.-May, and the other (intermolt breeding cycle) taking place only during intermolt (Aug.-Jan.); no breeding activity occurs during June-July. Results depicted in the present paper are also expected to help us evaluate the possible role of ecdysteroids in reproduction in crustaceans, a matter of active discussion during the past few years. Yolk deposition in the intermolt breeding cycle occurs under a very low ecdysteroid titer (3-6 ng/ml hemolymph), but it takes place at a perceptibly higher ecdysteroid titer (12-36 ng/ml hemolymph) in the premolt breeding cycle; a positive correlation exists between the ecdysteroid titer and oocyte diameter during the premolt breeding cycle. These results on the 1 hand indicate that yolk deposition can occur at a high ecdysteroid titer (premolt titer) in a natural population of *U. triangularis*; on the other hand, it reveals that a high ecdysteroid titer (as high as the premolt titer) is not mandatory for successful ovarian growth in the species. The study also suggests that molting and reproduction in the species are not mutually dependent events, as normal yolk deposition can occur irrespective of the molt stage. http://zoolstud.sinica.edu.tw/Journals/51.7/966.pdf

Key words: Decapod, Breeding, Growth, Hormone, Radioimmunoassay.

Crustaceans, unlike insects, continue to grow even after attaining puberty, a situation that makes the control of these processes more complex than in holometabolous insects. Breeding and molting in decapods are seasonal events, and their exact functioning is known to be accomplished through the up- and downregulation of inhibitory and stimulatory hormones (Adiyodi and Adiyodi 1970, Adiyodi 1988, Fingerman 1997, Nagaraju 2007 2011, Van Herp and Soyez 1997, Subramoniam 2011a). However, despite the vast body of literature available, endocrinologists are still unable to frame a common format for the integration of molting and reproduction for decapod crustaceans. This situation is constraining our understanding of the exact interplay of hormones which accomplishes these major physiological functions.

Having been established as growth hormones, ecdysteroids are also suspected to play pivotal

*To whom correspondence and reprint requests should be addressed. Tel: 91-9952187178; 91-416-2202575.
E-mail:ganilkumar@vit.ac.in

roles in crustacean reproduction (Subramoniam 2000 2011b, Diwan 2005, Hopkins 2009 for reviews). Although the role of ecdysteroids in insect reproduction was demonstrated beyond a doubt (Bloch et al. 2000, Martin et al. 2001, Hartfelder et al. 2002, Zhu et al. 2003, De Loof 2008, Dong et al. 2009, Swevers and Iatrou 2009), this aspect has not been adequately worked out in crustaceans, especially the brachyurans. This deficiency is primarily driven by the considerable diversity existing among various crustacean groups in integrating growth and reproduction. In palaemonid shrimp, yolk deposition and premolt growth are simultaneous events, wherein commencement of ovarian growth parallels the onset of premolt growth (Young et al. 1993, Okumura 2004). On the other hand, in most brachyuran species investigated so far, premolt growth and ovarian growth are mutually exclusive functions. For example, in the field crab *Paratelphusa hydrodromous* (Herbst 1794), an annual breeder, yolk deposition is a slow process with ovaries requiring approximately 4-5 mo to mature. During the breeding season (that commences by Nov./Dec.), the entire female population remains in intermolt until the spawning season (Apr./May), to be succeeded by the premolt/postmolt season (June/July). This pattern is typical of complete antagonism between molting and reproduction (Anilkumar 1980, Adiyodi 1988). Ovarian growth in the shore crab *Carcinus maenas* (Linnaeus 1758) is divided into 5 stages (stages 1-5), using oocyte diameter and color of the ovaries as criteria. Yolky ovaries are reported only during Apr.-June when the female population is in intermolt, exemplifying antagonism between molting and reproduction. An assay of ecdysteroids revealed the existence of a relatively low hemolymph titer in intermolt females that undergo vitellogenesis (Styrishave et al. 2004 2008). The estuarine crab *Metopograpsus messor* (Forskal 1775), a continuous breeder, the eggs of which mature in 14-16 d, arranges its premolt completely antagonistic to ovarian growth; ovaries of postmolt females; however, showed signs of yolk deposition, evincing postmolt-reproduction synergism (Sudha and Anilkumar 1996 2007, Syama et al. 2010). These reports of antagonism between premolt growth and reproduction imply that ecdysteroids may have a restraining influence on ovarian growth in these brachyuran crabs. On the contrary, in palaemonid shrimp, ovarian growth occurs concomitantly with premolt stages, suggesting an apparent gonad-stimulatory function of ecdysteroids (Okumura et al. 1992, Okumura and Aida 2000, Okumura 2004). It is at this juncture that we present results of our study that clearly demonstrate the simultaneous occurrence of premolt and reproduction in a brachyuran crab *Uca triangularis* (Milne-Edwards 1873), wherein premolt females undergo yolk deposition.

Inhabiting the intertidal region, this fiddler crab population exhibits discrete, seasonal patterns of growth and reproduction. This paper also depicts profiles of ecdysteroids related to stages of molt and reproduction, with a view to assessing possible impacts of the hormone on these physiological functions.

MATERIALS AND METHODS

All of the 1701 female fiddler crabs (*Uca triangularis*) used in the present study were collected from the intertidal zone of Muzhapilangad estuary (N. Kerala, India) by hand. Crabs were brought to the laboratory (approx. 8 km from the collection site) and maintained in cisterns made of plastic or cement. Sufficient care was taken to maintain them in near-natural conditions. Stages of ovarian growth were determined by observing the color of the ovaries and microscopic measurement of the oocyte diameter (OD). Oocyte samples were obtained by cutting open the carapace from the dorsal side using a sterilized pair of scissors. After being dissected out, oocytes were quickly rinsed in physiological saline and mounted on a glass slide, and their diameters were measured using an ocular micrometer. Accordingly, the process of ovarian growth in *U. triangularis* was classified into 3 stages. Early-stage ovaries (stage 1) appear yellow with an oocyte size of ≤ 90 μm. The ovary attains a brown hue at the mid-stage (stage 2) of its growth, with an oocyte diameter ranging 91-150 μm. As yolk deposition progresses towards stage 3, the ovary appears dark brown, with an oocyte diameter of 151-200 μm.

Molt stages were characterized by observing the setagenic events in the epipodite of the 3rd maxilliped (after Suganthi and Anilkumar 1999, Sudha and Anilkumar 2007). At intermolt (stage C_4), the epipodite epidermal layer is seen in close apposition to the cuticle. The onset of premolt (stage D_1) is marked by apolysis. Rudiments of setal grooves (denoting the formation of juvenile setae, a sign of stage D_2) appear within 9-11 d from the onset of D_1. The setal articulation and

setal cleft of juvenile setae become clearly visible (stage D_3) from 11-12 d after the onset of D_1. The setal tips become very distinct in early D_4, i.e., 12-13 days after D_1 has set in. In late D_4 (prior to ecdysis, i.e., about 14-15 d after the onset of D_1), juvenile setae can be seen extruding out of the setal groove. Exuviation proper (stage E) occurs within 15-16 d after the onset of D_1, and lasts for about 30-45 min. Early postmolt crabs (stage A that lasts for 1 d post-ecdysis) are easily distinguishable by the softness of their bodies and minimal activity (with no feeding); juvenile setae at this stage lack cones. By the 2nd-3rd day post-ecdysis (stage B), setal cones appear, the exoskeleton becomes hardened, and the crab begins feeding. As it approaches stage C (4-5 d post-ecdysis), the carapace greatly hardens, and the activity of the animal attains near-normalcy (after Supriya 2011).

To estimate the ecdysteroid titer through a radioimmunoassay (RIA), protocols used were based on Chang and O'Connor (1979) and subsequently modified as described in Sudha and Anilkumar (2007).

The biochemistry of the yolk components of *U. triangularis* at different stages of the molt and reproductive cycles was examined by the following methods. The total protein in tissues was precipitated by cold trichloroacetic acid (10%). The precipitate, separated out by centrifugation, was dissolved in 0.1 N NaOH, and was used as the protein extract for quantitative estimation using Folin-Ciocalteu's reagent (after Lowry et al. 1951, Anilkumar and Adiyodi 1980); bovine serum albumin (BSA) was used as the standard. In order to extract total lipids, tissues were homogenized with a chloroform: methanol (2: 1) mixture to a final volume 20 times that of the tissue sample. The homogenate was centrifuged (1800 xg); the supernatant was washed briefly with a 0.9% NaCl solution (by vortexing a few seconds) and centrifuged (1800 xg) again to separate the 2 phases. The lower chloroform phase (containing total lipids) was separated out and subjected to evaporation and a gravimetric analysis (Folch et al. 1957, Anilkumar and Adiyodi 1980). Carbohydrates of tissues were separated into an ethanol-soluble oligosaccharide fraction and an ethanol-insoluble polysaccharide fraction (after Johnston and Davies 1972), and estimated at 540 nm by the phenol sulfuric acid method (Dubois et al. 1956, Anilkumar and Adiyodi 1980). A portion of the ethanol-soluble fraction (prepared as described above) was also used to estimate the total free amino acids (after Lee and Takahashi 1966).

Levels of significance for statistical evaluations of data when necessary were determined using InStat Software (Graph Pad InStat, vers. 2.00, GraphPad Software, La Jolla California USA, www.graphpad.com)

RESULTS

Annual physiological cycle of molting and reproduction

The present data, resulting from weekly sampling of the female population of *U. triangularis*, revealed that the species is a continuous (multiparous) breeder, judged by the occurrence of growing ovaries in females that already held a brood in their broadened abdomen. Our close observations of breeding activities of the species further showed that ovarian maturation needs approximately 14-17 d. Spawned eggs required approximately 12-14 d of embryogenesis before larval release. In the meantime, the ovary is prepared for the next brood, to ensure spawning in 2-4 d after larval release.

The annual physiological cycle of *U. triangularis* is essentially comprised of 3 seasons of peak activity of breeding and/or molting. In Aug.-Jan., approximately 71% (556 of 788) of females were brood-carrying, while the remaining 29% (232 of 788) contained no berries but had growing ovaries (stages 2-3 of vitellogenesis). This would mean that almost all females of *U. triangularis* were engaging in reproductive activity in Aug.-Jan., when most of them were in intermolt. A small proportion of individuals (7 of 788 from our collection records; ~1%); however, were initiating premolt growth. Interestingly, all of the 7 premolt females were reproductively active (brood carrying and/or with growing ovaries), indicating simultaneous programming of premolt and reproduction (Table 1).

In Feb.-May, the entire female population (100%) of *U. triangularis* underwent breeding. While most of the females remained in intermolt, a subset of the (breeding) population underwent molting as well. The percentage with breeding activity; however, remained unchanged (compared to its frequency of Aug.-Jan.) during Feb.-Apr., but declined from May onwards. Of the total of 372 animals collected in Feb.-Apr., 303 (81.5%) were brood-carrying and 69 (18.5%) contained no

berries but had active ovaries; i.e., almost all of the female crabs were engaged in reproduction in Feb.-Apr. Of the 303 brood-carrying females, 22 were in premolt (6% of the total of 372), while 4 females were in postmolt (1% of the total). Nine of the 69 females containing no berries (2.4% of the total) were in premolt, while 17 females (4.6%) were in postmolt. Our examination of the population in Aug.-Apr. revealed that the majority of females were engaging in continuous release of their broods, one after the other, almost every 15 d, so that they released approximately 16-18 broods from Aug. to Apr. It is worth noting that the involvement of molting during Feb.-Apr. caused no reduction in the number of brood-carrying crabs, compared to those of Aug.-Jan. The percentage of breeding females; however, declined considerably in May. Of the 147 females collected during May, only 18 females were brood-carrying (12%); 16 of them were in intermolt, while 2 females were in premolt (1.4%). Among the remaining (129) females containing no berries (88%), 29 had maturing ovaries (19.7%), of which 19 (12.9%) were in intermolt, 1 (0.6%) was in premolt, and 9 (6%) were in postmolt. Of the remaining 100 non-breeding females (147 minus 47 = 100; 68%), 63 were in intermolt (43%), 22 females had reached premolt (15%), and 15 (10%) had reached postmolt. These 100 females had virtually entered a state of reproductive refractoriness that would last until the following Aug. It was further interesting to note that approximately 24% of the female population engaged in molting during June-July, while the remaining 76% engaged in neither molting nor reproduction (Tables 1, 2). In total, 394 female crabs were observed in June-July. Of these, 301 (76.4%) were in intermolt, while 56 (14.2%) and another 37 (9.4%) were in premolt and postmolt stages, respectively (Table 1). Ovaries of the entire population (all 394

Table 1. Table showing the seasonal occurrence of breeding activity (in percentage) of *Uca triangularis*

Seasons	Berried females with growing	Non-berried females ovaries (2)	Non-berried females without growing ovaries (3)	Breeding activity (%) (1)(1) + (2)
Aug. - Jan.	71 (0.5)[a]	29 (0.5)	Nil	100
Feb. - Apr.	81.5 (6)[1][b]	18.5 (2.4) [4.6]	Nil	100
#May	12 (1.4)	19.7 (0.6) [6]	68.3 (15) [10]	31.7
June - July	Nil	Nil	100 (14.2) [9.4]	Nil

[a]Values shown in parentheses represent premolt crabs (in percentage); [b]values in brackets are for postmolt crabs. #Being the transitory phase, we separated out data for the month of May.

Table 2. Monthly frequency of the occurrence of molt and reproduction in *Uca triangularis*

		Berried females		Non-berried females		Total number of Pre/ postmolt females (berried/ non- berried)
Month	N	IM	PM	IM	PM	
Jan.	130	110	0	20	0	0
Feb.	123	88	10[a]	18	7[a]	17[a]
Mar.	118	79	8[a]	17	14[a]	22[a]
Apr.	131	110	8[a]	8	5[a]	13[a]
May	147	16	2[a]	82	47(10)[a]	49 (12)[a]
June	187	0		152	35	35
July	207	0	0	149	58	58
Aug.	122	7	1[a]	112	2[a]	3[a]
Sept.	133	102	0	29	2[a]	2[a]
Oct.	124	87	0	36	1[a]	1[a]
Nov.	165	150	0	14	1[a]	1[a]
Dec.	114	96	0	18	0	0
Total Sample size	1701					

[a]Cases in which 100% of the premolt/postmolt females were engaged in reproductive activity. IM, intermolt; PM, premolt/postmolt females.

females) remained as white bands with no signs of yolk deposition. Frequencies of molting and reproduction in a given season were found to be easily discernible if data were collated in a monthly fashion, as shown in table 2.

Our assessment of the occurrence of molting and reproduction in females of *U. triangularis* revealed that they were essentially in intermolt until Jan., but showed signs of premolt initiation from Feb. onwards. Upon initiation of premolt (D_1), it takes about 15-16 d for a crab to undergo ecdysis proper (stage E). The percentage of premolt-postmolt individuals increased from approximately 10%-14% in Feb., to reach peak levels (33%) in May. Evidently, for this population of *U. triangularis*, mid-May is a transition month leading into the June-July molting period wherein the molting rate (~24%) is much greater than that of the Feb.-Apr. period (14%). We were further encouraged to parse out data on the May population, with a view to assessing the occurrence of molting among breeding females. This revealed that ~25% of breeding females in May (2/18 brood-carrying ones and 10/29 of ones without berries; 12/47 = ~25%) engaged in molting, a value very comparable to that of the June-July population. Thus, Aug.-Jan. is period of least molting activity (~1%), while May and June-July are periods of maximum molting activity (24%-25%), with Feb.-Apr. being in the middle (14%). Addressing the question of comparison of premolt-postmolt stages in Feb.-Apr. (premolt breeding cycle) and June-July (molting with no breeding cycle), we observed that molt stages (including substages) of both seasons were quite similar in characteristics and duration.

Hemolymph ecdysteroid titers at various stages of molting and breeding

An RIA of the hemolymph ecdysteroid titers was conducted on female *U. triangularis* during various seasons of the year. Our observations on the seasonal occurrence of molting and reproduction in the species revealed that the female population generally remained in intermolt during Aug.-Jan. During Feb.-Apr., on the other hand, several females (approximately 13%) entered premolt/postmolt activities, but exhibited active yolk deposition. This means that there could be 2 types of breeding cycles; i.e., one that takes place while females are in intermolt (intermolt breeding cycle), and the other while they are in premolt (premolt breeding cycle). While in the intermolt breeding cycle, the hemolymph ecdysteroid titer was at moderate/low levels without exhibiting any significant fluctuations throughout the entire duration of ovarian growth (Table 3). During the premolt breeding cycle; however, ovarian growth paralleled the progress of premolt growth and a concomitant increase in ecdysteroid titer. Special attention was given to this part of our study with a view to ascertaining if ovarian growth proceeds simultaneously with premolt growth (or else, if ovarian growth is arrested with the onset of premolt). For this purpose, the portion of the population that exhibited both premolt and ovarian growth during Feb.-Apr. was chosen. Significantly, (female) individuals in this entire portion of the population were highly synchronous with respect to the stages in ovarian growth and premolt growth. At the commencement of premolt (D_1), ovaries were in stage 1 (of maturation), and the concentration of hemolymph ecdysteroids was 12.77 ± 3.36 ng/ml. From then on, ecdysteroid levels showed a dramatic increase as animals reached D_3 (7-12 d from D_1) (26.00 ± 4.72 ng/ml) and D_4 (13-15 days from D_1) (34.00 ± 5.72 ng/ml) stages; ovaries of crabs at D_4 had reached stage 3, pending spawning (Tables 3, 4; Fig. 1). A statistical evaluation showed the existence of a positive correlation between oocyte diameter and the rising ecdysteroid titer in these females until the late premolt stage (D_4) ($p < 0.05$); the ecdysteroid titer

Table 3. Ecdysteroid levels (represented as ng/ml hemolymph; mean ± S.E.) in intermolt females of *Uca triangularis* undergoing vitellogenesis during Feb.-May (intermolt breeding cycle) (sample size in parentheses)

Reproductive stage	Oocyte diameter (in μm)	Ecdysteroid level
Stage 1	< 90	4.04 ± 1.33 (14)
Stage 2	91-150	3.01 ± 0.78 (13)
Stage 3	151-200	6.15 ± 1.59 (13)

declined from the postmolt stage onwards, when females appeared ready to spawn. Significantly, several of the females showed a tendency to raise another brood soon after spawning, evincing continuous breeding.

Ecdysteroid titer in females undergoing premolt but not ovarian growth

Ovaries of those females with no yolk deposition appeared as white bands. Our estimation of ecdysteroid levels of those females with no ovarian growth, but entering premolt, revealed that the hormone level increased in parallel with the progression of premolt growth. Furthermore, hemolymph ecdysteroid levels of premolt females undergoing the breeding cycle (premolt breeding cycle) (Table 4) were found to be comparable with those of (premolt) females with non-vitellogenic ovaries (Table 5).

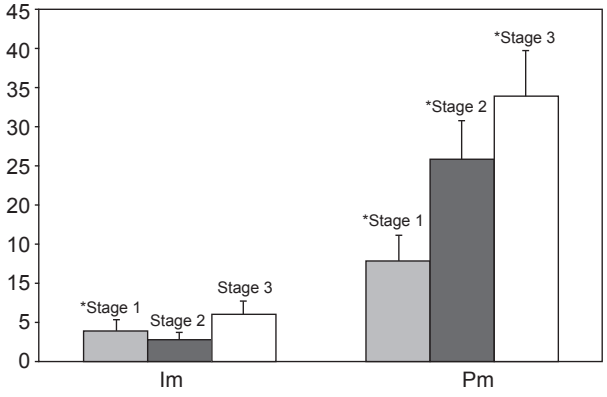

Fig. 1. Comparison of hemolymph ecdysteroid levels (Y-axis) (mean ± S.E.; ng/ml) in intermolt (Im) and premolt (Pm) breeding cycles of *Uca triangularis* with ovaries at stages 1-3. *Stage 1 (Im) < stage 1 (Pm); stage 1 (Im) < stage 2 (Im) < stage 3 (Im). * $p < 0.05$.

Ovarian biochemistry of intermolt and premolt breeding cycles

We examined ovarian biochemistry during the intermolt and premolt breeding cycles with a view to assessing the normalcy of ovarian growth in both instances. The major yolk components such as total proteins, carbohydrates (oligosaccharide and polysaccharide fractions), and total lipid contents of each stage of the ovary in an intermolt breeding cycle were found to be comparable to those of the respective ovarian stages of the premolt breeding cycle (Table 6), suggesting that yolk deposition, occurring in the intermolt breeding cycle (under a low profile of ecdysteroids), and the premolt breeding cycle (under relatively high ecdysteroid levels) were comparable with respect to yolk contents.

DISCUSSION

Our present observations reveal that *Uca triangularis* is multiparous in its breeding

Table 4. Ecdysteroid levels (ng/ml hemolymph; mean ± S.E.) in the premolt breeding cycle of *U. triangularis* during Feb.-May; spawning occurs in postmolt females (sample size in parentheses)

Molt stage	Ovarian stage	Ecdysteroid level
Early stage (D_0/D_1)	Stage 1	12.77 ± 3.36 (6)
Mid stage (D_2/D_3)	Stage 2	26.00 ± 4.72 (5)
Late stage (D_4)	Stage 2/ 3	34.00 ± 5.72 (6)
Postmolt (A/B)	Stage 3/ spawning	16.04 ± 3.41 (9)

Ecdysteroid levels: stage 1 < stage 2**; stage 2 < stage 3*. * $p < 0.05$; ** $p < 0.01$.

Table 5. Ecdysteroid levels (ng/ml hemolymph; mean ± S.E.) in premolt females of *Uca triangularis* not engaging in reproduction in June-July (sample size in parentheses)

Molt stage	D_0	D_1	D_2	D_3	D_4	E
Ecdysteroid titer	10.76 ± 3.96 (8)	16.00 ± 3.71 (6)	20.00 ± 4.32 (7)	31.55 ±2.74 (6)	33.46 ± 4.79 (9)	15.50 ± 2.66 (5)

pattern (releasing as many as 16-18 broods a year). The pattern shows a close resemblance to what was reported in the grapsid crab *Met. messor*, which releases 14-16 broods a year (Sudha and Anilkumar 1996), but differs from the Calicut population of the field crab *Paratelphusa hydrodromous* which is primiparous in nature (Anilkumar 1980, Adiyodi 1988). Multiparous breeding was reported in other fiddler crabs as well (Henmi 2003, Costa and Soares-Gomez 2009). The reproductive strategies (in terms of timing of larval release, mating, and spawning) adopted by multiparous females to ensure optimum viability of the sequence of egg clutches, have been worked out in brachyuran crabs (Paul et al. 1995, Paul and Paul 1996, Stevens 2003, Swiney 2008, Webb 2009). Mating in brachyurans can be either between a 'soft' postmolt female and a 'hard' intermolt male, or between a hard female and a hard male (Hartnoll 1969, DeGoursey and Auster 1992, Norman 1996, Raviv et al. 2008, Dittel and Epifanio 2009). In *U. triangularis*, mating takes place between a 'hard' female and a 'hard' male at various times of the year (Supriya et al. unpublished data). This prompted us to suspect that a single crab might be involved in mating more than once a year (i.e., 'multiparous mating'), apparently a strategy to ensure sufficient sperm to support multiparous breeding. The simultaneous existence of premolt growth and breeding activities observed in *U. triangularis*, judged by the presence of growing ovaries in almost all premolt females in Feb.-Apr., appears "atypical", if the results of previous investigations on other brachyuran crabs (Anilkumar 1980, Sudha and Anilkumar 1996, Styrishave et al. 2004 2008, Raviv et al. 2008, Syama et al. 2010) are considered the rule. The extent of concomitance between premolt growth and reproduction, exhibited by this population of *U. triangularis* appears more akin to what was described in the palaemonid shrimp *Macrobrachium rosenbergii* (De Man 1879) (Young et al. 1993, Okumura 2004, Sudha et al. 2010 2011), than other brachyuran crabs. The exact reason for such a "deviation" from the common brachyuran style of programming is not clear. The present study also enabled us to understand the occurrence of (exclusive) molting cycles with no involvement of breeding activity, taking place in *U. triangularis* in June and July. This situation appears to be comparable with the non-reproductive molting cycle of *Mac. rosenbergii* (Okumura and Aida 2000).

Our study also attempted to assess the possible impact of ecdysteroids in crustacean reproduction, a matter of active discussion during the past several years (Subramoniam 2000 2011b, Okumura 2004, Diwan 2005 for reviews). The gonadotropic role of ecdysteroids in eliciting the yolk-protein precursor (YPP) gene is well documented in insects (Zhu et al. 2003, Swevers and Iatrou 2009). Results of previous investigations argued for a stimulatory role of ecdysteroids in the reproduction of several non-brachyuran crustaceans like isopods (Steel and Vafopoulou 1998) and palaemonid shrimp (Okumura et al. 1992). Recently, 20-hydroxyecdysone was shown to stimulate HaVgI

Table 6. Comparison of ovarian biochemical reserves of intermolt and premolt females of *Uca triangularis* at stage 3 of vitellogenesis (weights represented as mg/100 g body weight; mean ± S.E.) (sample size in parentheses)

Biochemical reserves	Ovarian stage	Intermolt (Im)	Premolt (Pm)
Total protein	Stage 3 (NS)	1444.66 ± 28.45	1337.43 ± 79.18
	(t = 1.373; $p < 0.2$)	(6)	(5)
Total lipid	Stage 3 (NS)	1008.26 ± 18.12	961.46 ± 43.11
	(t = 1.069; $p < 0.15$)	(6)	(5)
Polysaccharide fraction	Stage 3 (NS)	114.88 ± 12.70	100.53 ± 6.97
	(t = 0.933; $p < 0.18$)	(5)	(5)
Oligosaccharide fraction	Stage 3 (NS)	113.56 ± 5.17	90.60 ± 17.51
	(t = 1.26; $p < 0.2$)	(5)	(5)
Total FAA	Stage 3 (NS)	36.71 ± 8.53	34.60 ± 4.12
	(t = 0.223; $p < 0.83$)	(6)	(5)

NS indicates that there is no statistically significant difference in the ovarian biochemistry between Im and Pm females.

expression in ovaries of the American lobster *Homarus americanus* (Tiu et al. 2009). However, a stimulatory role of ecdysteroids in brachyuran vitellogenesis has yet to be demonstrated (see Subramoniam 2011a and b for reviews). Interestingly, ovarian growth during the premolt breeding cycle in *U. triangularis* proceeds under a perceptibly high hemolymph ecdysteroid titer (Fig. 1), comparable to the situation in the palaemonid shrimp *Mac. rosenbergii* (Young et al. 1993, Okumura 2004, Sudha et al. 2010 2011). The progress of yolk deposition from stages 1 to 3, coupled with an escalating ecdysteroid titer (from ~4 to ~34 ng/ml hemolymph; $p < 0.05$), is closely entrained with premolt growth (from D_1 to D_4) (Table 3). These results demonstrated that a relatively high molting hormone titer (premolt titer for the species) would not impede ovarian growth in *U. triangularis*. It would also be worth noting at this juncture that the percentage of crabs carrying broods was almost the same (~70%-80%) in both Aug.-Jan. (when ecdysteroids in the population are generally at low levels) and in mid-Feb.-May seasons (when the molting frequency is at its maximum). Further, it is tempting to ask whether the ecdysteroids at a premolt titer are a prerequisite for successful ovarian maturation in *U. triangularis*. The occurrence of the intermolt breeding cycle (Aug.-Jan.) of *U. triangularis* under a low concentration of ecdysteroids (Table 3) reveals that the premolt titer of ecdysteroids is not mandatory for accomplishing successful yolk deposition in this species. It is also very pertinent to note that ecdysteroid levels in *U. triangularis* (present study) were considerably lower than what were reported for other brachyurans, in which premolt and ovarian growth are mutually exclusive events. Our laboratory previously performed an ecdysteroid assay in female populations of the brachyuran crab *Met. messor* (Sudha and Anilkumar 2007), wherein the intermolt levels of ecdysteroids were 10-30 ng/ml hemolymph (vs. 4-6 ng/ml in *U. triangularis*). The onset of premolt resulted in an increase in ecdysteroid levels in both *Met. messor* (~45 ng/ml) and *U. triangularis* (~12-13 ng/ml). As premolt proceeded towards D_4, levels reached ~170 ng/ml in *Met. messor* and ~35 ng/ml in *U. triangularis* (present study). Interestingly, we found that ecdysteroid levels of *U. triangularis* were comparable to "reproductive molt cycles" reported in *Mac. rosenbergii*. Significantly, both *U. triangularis* (present study) and *Mac. rosenbergii* (Okumura and Aida 2000) share a common feature of synergistic occurrence of premolt growth and yolk deposition, a striking deviation from the majority of brachyurans. It should, however, be noted that the exact cause of a discrepancy in ecdysteroid levels between *U. triangularis* and other brachyurans (including *Met. messor*) is still enigmatic.

The molt cycles which *U. triangularis* undergoes in various seasons (Feb.-May and June-July) of the year were comparable to hemolymph ecdysteroid levels (Tables 4, 5). However, some discrepancy exists between these 2 molt cycles (of Feb.-May and June-July) in that in the former, premolt growth is concomitant with active vitellogenesis, while in the latter, none of the females possessed growing ovaries. This situation means that fluctuating ecdysteroid levels, as seen in the Feb.-May population (Table 3) are primarily necessary to accomplish successful ecdysis, but might not be a prerequisite for ovarian growth. Moreover, yolk deposition during the intermolt breeding cycle (under a relatively low ecdysteroid titer; Table 3; Fig. 1) appears quite comparable to that of the premolt breeding cycle (under an elevated ecdysteroid titer, as the crab proceeds from stages D_1 to D_4) in terms of the duration of vitellogenesis, oocyte size, and ovarian biochemistry (Table 6). This observation further supports our view that an elevated titer (as high as the premolt titer) of ecdysteroids is not a requisite for successful ovarian maturation.

This could also reveal that these 2 functions (molting and reproduction), at least in their final phases of manifestation, are not mutually dependent; normal yolk deposition occurs irrespective of whether it is a premolt or intermolt breeding cycle. Recent investigations conducted on the desert locust *Schistocerca gregaria* are reminiscent of a possible dual function for ecdysteroids. In this locust, the role of the Halloween genes (spook (spo) and phantom (phm)) in upregulating ecdysteroid synthesis was shown by expression studies. Interestingly, despite downregulation of these genes with RNA interference (RNAi), and the resultant reduction in ecdysteroid levels, molting continued (Marchal et al. 2011). This phenomenon poses a crucial question regarding the close entrainment between molting and ecdysteroid levels. On the other hand, it also led us to suspect that the ecdysteroid peak may serve another function than has been assumed hitherto.

In conclusion, the present paper, for the 1st time, clearly demonstrates how premolt growth and reproduction are concomitantly accommodated

in the annual cycle of a brachyuran crab (*U. triangularis*). On the 1 hand, the population exhibits an intermolt breeding cycle, akin to other brachyuran crabs that exhibit antagonistic programming of molting and reproduction. On the other hand, during Feb.-Apr./May, the population accommodates ovarian growth and premolt growth simultaneously in a pattern comparable to palaemonid shrimp (*Mac. rosenbergii*, for instance). Further, the molting cycle which this species undergoes, that is devoid of a reproductive cycle, appears comparable to the non-reproductive molt cycle in *Mac. rosenbergii* (Okumura and Aida 2000). Our close examination of the molting-reproductive interaction in *U. triangularis* in relation to various seasons further revealed that at least in the final phases, the manifestation of these functions could be independent of the other: accomplishing one (molting, for example) might not be dependent on the programming of the other (ovarian growth). Addressing the question of ecdysteroids and reproduction, we are not certain as to what extent ecdysteroids influence yolk deposition in *U. triangularis*. Interestingly, we found a positive correlation between ecdysteroid titers and oocyte size during Feb.-Apr., when several females were engaged in both molting and reproduction. Nevertheless, this correlation could not be taken as sufficient grounds to advocate for the role of ecdysteroids in reproduction in the species. This observation, however, allows us to state that a high (premolt) ecdysteroid titer does not have a restraining influence on reproduction (as implied by the existence of antagonism between molting and reproduction in several brachyuran crabs reported so far). Another notable fact in this context was the presence of a low titer of ecdysteroids in intermolt females (Aug.-Jan.) that exhibited ovarian growth. It would certainly be worth exploring whether this low titer of ecdysteroids plays a crucial role in accomplishing successful ovarian maturation during the season. The role of ecdysteroids as a gonadotropic hormone that stimulates vitellogenin synthesis in fat bodies of insects was demonstrated. Recent investigations also revealed ecdysteroid-mediated signaling related to insect vitellogenesis at the molecular level (Bloch et al. 2000, Martin et al. 2001, Hartfelder et al. 2002, Zhu et al. 2003, Dong et al. 2009). Such a clear demonstration of the sequence of events involving ecdysteroids in triggering yolk synthesis is, however, yet to be demonstrated in brachyuran crustaceans. The ecdysteroid receptor gene (*EcR*) was cloned in the fiddler crab *U. pugilator* (Bosc 1802) (Durica et al. 2002) and the land crab *Gecarcinus lateralis* (Freminville 1835) (Kim et al. 2005). Gene expression studies clearly demonstrated the occurrence of EcR messenger RNA in ovarian tissues, implying that the ovaries could be a target for ecdysteroids in crustaceans (Durica et al. 2002, Kim et al. 2005, Hopkins et al. 2008). That ecdysteroids can trigger the cascade of events leading to successful ovarian maturation in crustaceans is a matter to be probed through future research.

Acknowledgments: KS and GA gratefully acknowledge the financial support from the International Foundation for Science (Stockholm, Sweden) (RGA: A/3520-1) and the Department of Atomic Energy (Government of India), respectively. NTS thanks Kannur Univ. for the award of a Junior Research Fellowship during the tenure of this work. Thanks are also due to the Amala Cancer Research Centre (Trichur, India) for providing a liquid scintillation counter.

REFERENCE

Adiyodi KG, RG Adiyodi. 1970. Endocrine control of reproduction in decapod crustaceans. Biol. Rev. **45:** 121-165.

Adiyodi RG. 1988. Reproduction and development. *In* WW Burggren, BR McMahon, eds. Biology of land crabs. New York: Cambridge Univ. Press, pp. 139-185.

Anilkumar G. 1980. Reproductive Physiology of female crustaceans. PhD dissertation, University of Calicut, Calicut, India.

Anilkumar G, KG Adiyodi. 1980. Ovarian growth induced by eyestalk ablation during the prebreeding season is not normal in the crab, *Paratelphusa hydrodromous* (Herbst). Int. J. Invert. Reprod. **2:** 95-105.

Bloch G, DW Borst, ZY Huang, GE Robinson, J Cnaani, A Hefetz. 2000. Juvenile hormone titers, juvenile hormone biosynthesis, ovarian development and social environment in *Bombus terrestris*. J. Insect Physiol. **46:** 47-57.

Chang ES, JD O'Connor. 1979. Arthropod molting hormones. *In* BM Jaffe, HR Behrman, eds. Methods of hormone radioimmunoassay. New York: Academic Press, pp. 797-814.

Costa T, A Soares-Gomez. 2009. Population structure and reproductive biology of *Uca rapax* (Decapod: Ocypodidae) in a tropical coastal lagoon, southeast Brazil. Zoologia **26:** 647-657.

De Loof A. 2008. Ecdysteroids, juvenile hormone and insect neuropeptides: recent successes and remaining challenges. Gen. Compar. Endocrinol. **155:** 3-13.

DeGoursey RE, PJ Auster. 1992. A mating aggregation of the spider crab (*Libinia emarginata*). J. Northwest Atlantic Fish. Sci. **13:** 77-82.

Dittel AI, CE Epifanio. 2009. Invasion of Chinese mitten crab

Eriochier sinensis: a brief review. J. Exp. Mar. Biol. Ecol. **374:** 79-92.

Diwan AD. 2005. Current progress in shrimp endocrinology - a review. Indian J. Exp. Biol. **43:** 209-223.

Dong SZ, GY Ye, JY Guo, C Hu. 2009. Role of ecdysteroid and juvenile hormone in vitellogenesis in an endoparasitic wasp, *Pteromalus puparum* (Hymenoptera: Pteromalidae). Gen. Compar. Endocrinol. **160:** 102-108.

Dubois M, KA Gills, JK Hamilton, PA Rebers, F Smith. 1956. Colorimetric methods for determination of sugars and related substances. Anal. Chem. **28:** 350-356.

Durica DS, X Wu, G Anilkumar, PM Hopkins, AC-K Chung. 2002. Characterization of crab EcR and RXR homologs and expression during limb regeneration and oocyte maturation. Mol. Cell. Endocrinol. **189:** 59-76.

Fingerman M. 1997. Crustacean endocrinology: a retrospective, prospective, and introspective analysis. Physiol. Zool. **70:** 257-269.

Folch J, M Lees, GH Sloane-Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. **266:** 497-509.

Hartfelder K, MMG Bitondi, WC Santana, ZLP Simoes. 2002. Ecdysteroid titre and reproduction in queens and workers of the honey bee and of a stingless bee: loss of ecdysteroid function at increasing levels of sociality. Insect Biochem. Mol. Biol. **332:** 211-221.

Hartnoll RG. 1969. Mating in the Brachyura. Crustaceana **16:** 161-181.

Henmi Y. 2003. Trade-off between brood size and brood interval and the evolution of underground incubation in three fiddler crabs (*Uca perplexa*, *U. vocans*, and *U. dussumieri*). J. Crust. Biol. **23:** 46-54.

Hopkins PM. 2009. Crustacean ecdysteroids and their receptors. *In* G. Smagghe, ed. Ecdysone: structures and functions. New York: Springer, pp. 73-98.

Hopkins PM, DS Durica, T Washington. 2008. RXR isoforms and endogenous retinoids in the fiddler crab, *Uca pugilator*. Compar. Biochem. Physiol. Part A **151:** 602-614.

Johnston MA, PS Davies. 1972. Carbohydrates of the hepatopancreas and blood tissues of the *Carcinus*. Compar. Biochem. Physiol. **41B:** 433-443.

Kim HW, ES Chang, DL Mykles. 2005. Three calpains and ecdysone receptor in the land crab *Gecarcinus lateralis*: sequences, expression and effects of elevated ecdysteroid induced by eyestalk ablation. J. Exp. Biol. **208:** 3177-3197.

Lee YP, T Takahashi. 1966. An improved colorimetric determination of amino acids with the use of ninhydrin. Anal. Biochem. **14:** 71-77.

Lowry OH, NJ Rosebrough, AL Farr, RJ Randall. 1951. Protein measurement with Folin-phenol reagent. J. Biol. Chem. **193:** 265-275.

Marchal E, L Badisco L, H Verlinden, T Vandersmissen, SV Soest, PV Weilendaele, JV Broeck. 2011. Role of the Halloween genes, spook and phantom in ecdysteroidogenesis in the desert locust, *Schistocerca gregaria*. J. Insect Physiol. **57:** 1240-1248.

Martin D, SF Wang, AS Raikhel. 2001. The vitellogenin gene of the mosquito *Aedes aegypti* is a direct target of ecdysteroid receptor. Mol. Cell. Endocrinol. **173:** 75-86.

Nagaraju GPC. 2007. Is methyl farnesoate a crustacean hormone? Aquaculture **272:** 39-54.

Nagaraju GPC. 2011. Reproductive regulators in decapod crustaceans: an overview. J. Exp. iol. **214:** 3-16.

Norman CP. 1996. Reproductive biology and evidence for hard-female mating in the brachyuran crab *Thalamita sima* (Portunidae). J. Crust. Biol. **16:** 656-662.

Okumura T. 2004. Perspectives on hormonal manipulation of shrimp reproduction. Jpn. Agric. Res. **38:** 49-54.

Okumura T, K Aida. 2000. Fluctuations in hemolymph ecdysteroid levels during the reproductive and non-reproductive molt cycles in the giant freshwater prawn *Macrobrachium rosenbergii*. Fish. Sci. **66:** 678-685.

Okumura T, CH Han, Y Susuki, K Aida. 1992. Changes in haemolymph vitellogenin and ecdysteroid levels during the reproductive and non-reproductive molt cycles in the giant freshwater prawn *Macrobrachium rosenbergii*. Zool. Sci. **9:** 37-45.

Paul AJ, JM Paul. 1996. Observations on mating of multiparous *Chionoecetes bairdi* Rathbun (Decapoda: Majidae) held with different sizes of males and one-clawed males. J. Crust. Biol. **16:** 295-299.

Paul AJ, JM Paul, WE Donaldson. 1995. Shell condition and breeding success in Tanner crabs. J. Crust. Biol. **15:** 476-480.

Raviv S, S Parnes, A Sagi. 2008. Coordination of reproduction and molt in decapods. *In* E. Mente, ed. Reproductive biology of crustaceans. Enfield, NH: Science Publishers, pp. 365-390.

Steel CGH, X Vafopoulou. 1998. Ecdysteroid titers in hemolymph and other tissues during molting and reproduction in the terrestrial isopod, *Oniscus ascellus* (L.). Invert. Reprod. Develop. **34:** 187-194.

Stevens BG. 2003. Timing of aggregation and larval release by Tanner crabs, *Chionoecetes bairdi*, in relation to tidal current patterns. Fish. Res. **65:** 201-216.

Styrishave B, K Rewitz, T Lund, O Andersen. 2004. Variations in ecdysteroid levels and cytochrome p450 expression during moult and reproduction in male shore crabs *Carcinus maenas*. Mar. Ecol. Progr. Ser. **274:** 215-224.

Styrishave B, T Lund, O Andersen. 2008. Ecdysteroids in female shore crabs *Carcinus maenas* during the moulting cycle and oocyte development. J. Mar. Biol. Assoc. UK **88:** 575-581.

Subramoniam T. 2000. Crustacean ecdysteroids in reproduction and embryogenesis. Compar. Biochem. Physiol. Part C **125:** 135-156.

Subramoniam T. 2011a. Mechanisms and control of vitellogenesis in crustaceans. Fish. Sci. **77:** 1-21.

Subramoniam T. 2011b. In search of a gonadotropic hormone in Crustacea. Curr. Sci. **101:** 18-19.

Sudha K, G Anilkumar. 1996. Seasonal growth and reproduction in a highly fecund crab, *Metopograpsus messor* (Forskal) (Grapsidae). Hydrobiologia **319:** 15-21.

Sudha K, G Anilkumar. 2007. Elevated ecdysteroid titre and precocious molt and vitellogenesis induced by eyestalk ablation in the estuarine crab, *Metopograpsus messor* (Brachyura: Decapoda). J. Crust. Biol. **27:** 304-308.

Sudha K, G Anilkumar, JH Brown. 2011. Ecdysteroid levels related to growth and reproduction in the freshwater prawn *Macrobrachium rosenbergii*. Proceedings Asia-Pacific Aquaculture 2011, Kochi, India.

Sudha K, NT Supriya, V Krishnakumar, G Anilkumar, JH Brown. 2010. Ecdysteroid levels in three decapod species in relation to growth and reproduction: comparative study. Proceedings 7th International Crustacean Congress (ICC7), Qingdao, China, 20-25 June 2010, p. 264.

Suganthi AS, G Anilkumar. 1999. Molt-related fluctuation in ecdysteroid titre and spermatogenesis in the crab, *Metopograpsus messor* (Brachyura: Decapoda). Zool. Stud. **38:** 313-320.

Supriya NT. 2011. Studies on hormonal regulation and storage physiology of growth and reproduction in decapod crustaceans. PhD dissertation, Kannur Univ., Kannur, India.

Swevers L, K Iatrou. 2009. Ecdysteroids and ecdysteroid signaling pathways during insect oogenesis. *In* G Smagghe, ed. Ecdysone: structures and functions. New York: Springer, pp. 127-164.

Swiney KM. 2008. Egg extrusion, embryo development, timing and duration of eclosion, and incubation period of primiparous and multiparous tanner crabs (*Chionoecetes bairdi*). J. Crust. Biol. **28:** 334-341.

Syama VP, NT Supriya, K Sudha, G Anilkumar. 2010. Seasonal growth and reproduction in two brachyuran species inhabiting diverse ecosystems. *In* VK Gupta, AK Verma, JD Singh, eds. Perspectives in animal ecology and reproduction, Vol. VI. New Delhi: Daya Publications, pp. 275-289.

Tiu SH, HL Hui, B Tsukimura, SS Tobe, JG He, SM Chan. 2009. Cloning and expression study of the lobster (*Homarus americanus*) vitellogenin: conservation in gene structure among decapods. Gen. Compar. Endocrinol. **160:** 36-46.

Van Herp F, D Soyez. 1997. Arthropoda-Crustacea. *In* KG Adiyodi, RG Adiyodi, eds. Progress in reproductive endocrinology, Vol. VIII. Reproductive biology of invertebrates. New York: Wiley, pp. 247-275.

Webb JB. 2009. Reproductive success of multiparous female tanner crab (*Chionoecetes bairdi*) fertilizing eggs with or without recent access to males. J. Northwest Atlantic Fish. Sci. **41:** 163-172.

Young, NJ, SG Webster, HH Rees. 1993. Ovarian and hemolymph ecdysteroid titres during vitellogenesis in *Macrobrachium rosenbergii*. Gen. Compar. Endocrinol. **90:** 183-191.

Zhu LS, K Miura, L Chen, AS Raikhel. 2003. Cyclicity of mosquito vitellogenic ecdysteroid-mediated signaling is modulated by alternative dimerization of the RXR homologue Ultraspiracle. Proc. Natl. Acad. Sci. USA **100:** 544-549.