

Moult-related Fluctuation in Ecdysteroid Titre and Spermatogenesis in the Crab, *Metopograpsus messor* (Brachyura: Decapoda)

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Arundhathi Shyla Suganthi and Gopinathan Anilkumar (1999) Moult-related fluctuation in ecdysteroid titre and spermatogenesis in the crab, *Metopograpsus messor* (Brachyura: Decapoda). *Zoological Studies* 38(3): 314-321. The present study examines the interrelation between growth and reproduction in males of the brachyuran crab, *Metopograpsus messor*. Molt stages were characterized through observing the setal development of the maxilliped epipodite. During intermolt, the testis of *M. messor* shows the presence of various spermatogenic stages (such as spermatogonia, spermatocytes, spermatids, and spermatozoa) in almost equal proportions. However, the onset of premolt (coupled with a significant rise in ecdysteroid titre) results in a significant increase in spermatogonial proliferation and refractoriness in sperm maturation. Our results also reveal that at least a low level of ecdysteroid is present during the intermolt stage, when spermatogenesis occurs normally.

Key words: Crustacea, Epipodite, Growth, Reproduction, Spermatozoa.

Programming of growth and reproduction has been extensively investigated in female decapods. Brachyuran crabs are known to undergo vitellogenesis only when they are not in the premolt stage (Adiyodi 1988, Sudha and Anilkumar 1996). Conversely, in natantian prawns, growth and ovarian maturation are synergistic events (Wilder et al. 1991, Okumura et al. 1992, Wilder and Aida 1995). Reports are also available which strongly suggest that in insects the ecdysteroids influence not only growth but reproduction as well (Hoffmann et al. 1980, Raabe 1989, Grieneisen 1994, Bocking et al. 1995). The presence of ecdysone and 20-hydroxyecdysone is also suggested to stimulate spermatogenesis in many insect species (Loeb 1991, Zhang et al. 1995). However, information is lacking with respect to the intricate balancing between somatic growth and reproduction in male brachyurans. With this in mind, we have examined in the present study the spermatogenic activity of the brachyuran crab, *Metopograpsus messor*, displaying varying titres of ecdysteroid, in relation to moult stages. In order to characterize the moult stages, earlier workers em-

ployed several methods which were primarily based on such features as carapace color, measurements of tissue ash and glycogen, and histological observations of the integument (Schwabe et al. 1952, Waterman 1960, Stevenson 1972, Ennis 1973, Haefner and Van Engel 1975). These methods, however, tend to be cumbersome, especially for evaluating a large sample size. Subsequently, some authors have identified the sequential events in setal development of appendages such as pleopods and uropods (of either brachyuran or non-brachyuran females) to characterize moult stages (Peebles 1977, Anilkumar 1980, Lyle and MacDonald 1983, Sreekumar and Adiyodi 1983, Sudha and Anilkumar 1996, Vijayan et al. 1997). However, pleopods in male brachyurans are degenerate structures, and hence, they are not suitable for identification of moult stages. Furthermore, the brachyuran decapods are devoid of uropods. In *Chionoecetes opilio*, the majid crab, setal development of the maxilliped exopodite was used to delineate moult stages (O'Halloran and O'Dor 1988). The maxilliped exopodite of *M. messor* (a grapsid crab), however, was found to be so slen-

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der that setal development was not discernible. On the other hand, its (maxilliped) epipodite, being sufficiently broad and transparent to allow precise observation of the moult-related events in setal development, is used in the present study as a tool to accurately assess moult stages.

MATERIALS AND METHODS

Adult males of *Metopograpsus messor*, carapace width ranging between 1.8 and 2.2 cm, were collected during Mar.-Apr. 1993 from intertidal regions of the Muzhupilangad estuary (Cannanore, India). Animals were collected through baiting. They were maintained for 2-3 d in the laboratory in plastic cisterns filled with wet sand and were fed ad libitum on clam meat.

In the present study, 48 crabs were used in order to assess, in detail, the events in setal development of the maxilliped epipodite in relation to moult stages. The epipodite of the 3rd maxilliped was carefully removed using a pair of scissors, placed on a clean glass slide, and microscopically observed.

Fifteen crabs, 5 each from the intermoult, premoult, and postmoult stages, were used to study ecdysteroid titre and spermatogenesis. Prior to the ecdysteroid assay, each crab was weighed, and the total haemolymph volume was measured. For histological preparation, testes were dissected out in 0.9% normal saline (after Oser 1965). The tissue was fixed in Carnoy's fixative for 45 min after which it was dehydrated in ethanol grades. Paraffin sections 5-7 μm thick were stained with Harris haematoxylin and 1% alcoholic eosin (Humason 1967). Size and chromatin nature of the differentiating gametes (after Gupta et al. 1989, Minagawa et al. 1994) were used in the present study as the criteria for determining the stages in spermatogenic activity.

The compounds that bind immunochemically with the antisera prepared against 2-succinyl conjugate of ecdysone (obtained as a gift from Dr. Bollenbacher, Code A-2) are referred to as ecdysteroids in the present study.

Haemolymph ecdysteroids levels were estimated through radioimmunoassay by the method of Chang and O'Connor (1979). Fresh haemolymph (10 μl) was collected in vials containing 100 μl borate buffer (0.1 M boric acid, 0.1 M sodium tetraborate, 0.075 M sodium chloride, pH 8.4) and the radioligand, ^3H -ecdysone (approximately 12 000 dpm) (NEN, City, ST). The standards consisting of ^3H -ecdysone, buffer, and the standard ecdysone (Sigma, City, ST) were prepared along with the

(unknown) samples and vortexed to allow adequate mixing. To each vial, 100 μl of antiserum was added. The mixture was incubated for 12 h at 4 °C. After incubation, 5 μl of the control rabbit serum was also added to each vial, and mixed well, after which 200 μl saturated $(\text{NH}_4)_2\text{SO}_4$ was added into each vial and kept at 4 °C for 20 min. The vials were subsequently centrifuged at 4800 g for 20 min. The pellets were washed with 400 μl of 50% $(\text{NH}_4)_2\text{SO}_4$ in borate buffer and centrifuged. The pellets were dissolved in 25 μl distilled water. Then, 500 μl of the scintillation cocktail (prepared by dissolving 0.8% PPO and 0.02% POPOP in a toluene:triton [2: 1] mixture) was added, and the samples were placed into scintillation vials and read using an LKB Liquid Scintillation Counter. Various concentrations (50, 100, 200, 400, 800, and 1600 pg) of standard ecdysone were used for plotting the standard curve.

For the purpose of statistical evaluation, data on ecdysteroid titre and percentage of spermatids at various moult stages were subjected to multiple comparisons after ANOVA (post tests). Further, the Tukey-Kramer multiple comparison test was employed to assess *p* values for comparison between each pair of columns. As there were only 2 variables, *t*-test was used to assess the levels of significance of differences in percentage of spermatogonial cells in intermoult and premoult stages (Croxtton 1953, Sokal and Rohlf 1981). The analyses were carried out using InStat software (GraphPad InStat, Version 2.00, 1993) on a Pentium computer.

RESULTS

Identification of moult stages through microscopic observation of maxillipeds

During the intermoult stage, the epidermal layer of the maxilliped epipodite is seen closely adjoined to the cuticle (Fig. 1), and the carapace appears hard and thick. The onset of premoult (D_1) is characterised by epidermal retraction and the appearance of rudiments of setal grooves from the epidermal layer; the setal grooves become more prominent during D_2 . At D_3 , the setal articulation of the juvenile setae and the setal cleft are clearly visible; the setae are seen discretely arranged within the setal groove. A new cuticular layer is formed over the retracted epidermis (Fig. 2). During the D_4 stage, the carapace appears so thin that slight pressure may cause it to break. The setal tips become more distinct in the early D_4 stage (Fig. 3). Prior to ecdysis (late D_4), the juvenile setae are extruded out of the setal grooves

(Fig. 4). The process of exuviation lasts about 30-45 min. Epipodites of early post-ecdysial crabs appear transparent and exhibit the following distinct features: (1) the setal lumen is continuous with the setal base until the appearance of the setal cone, 24 h after ecdysis; and (2) the cuticle lacks mineralization and hence appears thin compared to that of intermoult crabs ($t = 7.46$; $p < 0.001$). Subsequently, after another 5-6 d, the exoskeleton undergoes chitinization, resulting in thickening of the epipodite cuticle to intermoult levels.

Moult-related changes in ecdysteroid titre

The ecdysteroid titre in the haemolymph of intermoult crabs was found to be 126.10 ± 23.76 ng/100 g body weight (mean \pm SD). The onset of premoult (D_1 - D_2) resulted in a dramatic rise ($p < 0.001$) in the ecdysteroid levels that lasted until exuviation. The ecdysteroid level of postmoult animals (within 24 h postecdysis) was 165.04 ± 23.89 ng/100 g body weight, which was significantly lower ($p < 0.01$) than that of the premoult stage (Table 1). Analysis of variance showed that the variations in the haemolymph ecdysteroid levels related to the various moult stages are extremely significant ($F = 21.484$; $p < 0.0001$).

Stages in spermatogenesis

The male reproductive system of *M. messor* consists of a pair of highly coiled, tubular testes extending dorso-laterally along the cephalothorax. In animals with 1.8-2.2 cm carapace width, the testicular tubule, if unwound, would measure 7-9 cm in length. Testes of both sides are fused distally and lead into the vasa deferentia and ejaculatory duct.

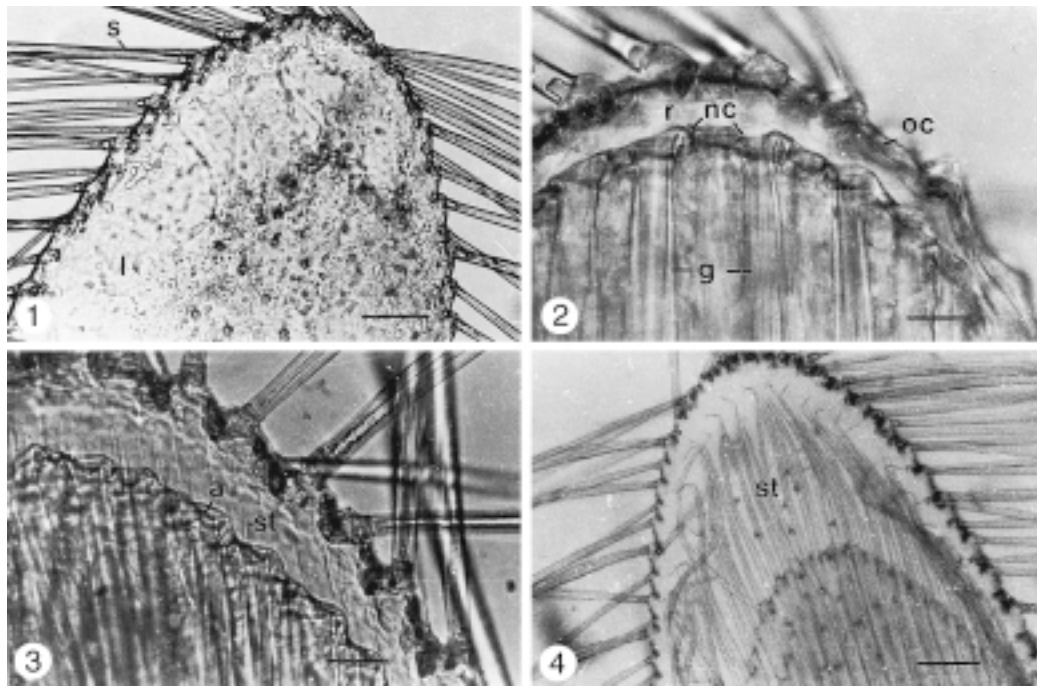
The testicular acini of *M. messor* possess an outer connective tissue layer, and an inner epithelial layer which ranges in height from 4.78 to 9.56 μm .

Table 1. Ecdysteroid levels during different moult stages of male *Metopograpsus messor* (weights represented in ng/100 g body weight) (mean \pm SD) (Sample size in parantheses)

Moult stage	Ecdysteroids
Intermoult (5)	126.10 ± 23.76
Premoult (5)	240.70 ± 35.15
Postmoult (5)	165.04 ± 23.89

($F = 21.484$) ($p < 0.0001$)

As per Tukey-Kramer multiple comparison test: premoult titre > intermoult titre ($p < 0.001$); premoult titre > postmoult titre ($p < 0.01$).



Figs. 1-4. Maxilliped epipodite of *Metopograpsus messor* in relation to moult stages. 1. Intermoult stage; scale = 300 μm ; 2. D_2 stage; scale = 40 μm ; 3. Early D_4 stage; scale = 50 μm ; 4. Late D_4 stage; scale = 250 μm .

Legend: a, setal articulation; g, setal grooves; l, lumen; nc, new cuticle; oc, old cuticle; r, retracted area; s, setae; st, setal tips.

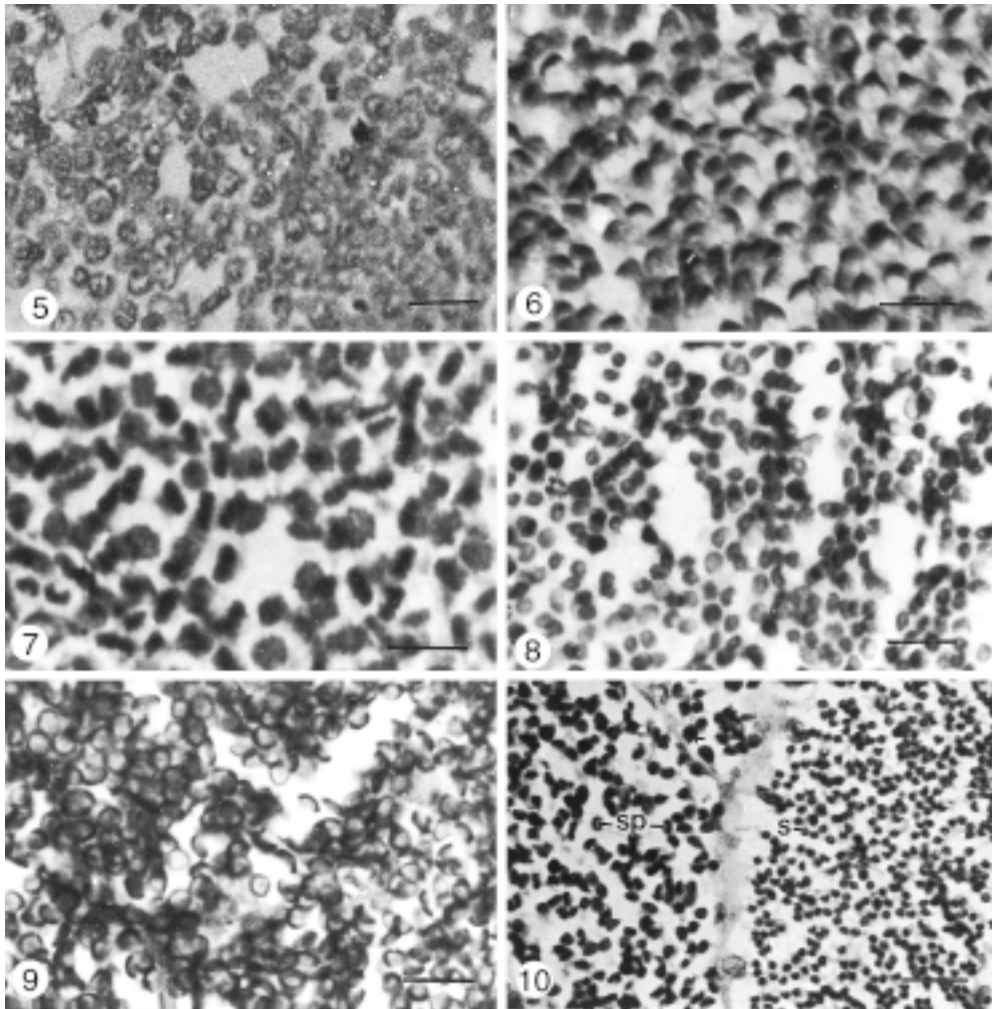
The acini encompass the germ cells at various stages of proliferation and maturation:

- (i) *Spermatogonia*: Spherical cells are about 5-7 μm in diameter; the nucleo-cytoplasmic ratio is 0.83 ± 0.06 (Fig. 5).
- (ii) *Spermatocytes*: The primary spermatocytes (4 μm in diameter) appear as crescent-shaped structures due to the characteristic chromatin clumping (Fig. 6). Secondary spermatocytes show chromatin grouping reminiscent of the 2nd meiotic division (Fig. 7).
- (iii) *Spermatids*: Each spermatid measures about 3 μm in diameter. Under the light microscope, spermatids and the chromatin within appear ring-like (Fig. 8).

- (iv) *Spermatozoa*: The size of the spermatozoa is comparable with that of the spermatids. Under the light microscope, the spermatozoa appear crescent shaped, with a cup-shaped nucleus that partially encompasses the vesicular acrosome (Fig. 9).

Moult-related changes in spermatogenic activity

Intermoult: Active spermatogonial proliferation occurs during intermoult. The proliferative spermatogonia comprise about 16% of the total germ cells (relative number per unit area) in the intermoult stage. The primary and secondary spermatocytes constitute about 24% and 20%, respectively, of the total germ cells, while the spermatids and the sper-



Figs. 5-10. Section of testicular acini of *Metopograpsus messor* showing spermatogenic stages (haematoxylin-eosin). **5.** Spermatogonia; scale = 100 μm ; **6.** Primary spermatocytes; scale = 10 μm ; **7.** Secondary spermatocytes; scale = 10 μm ; **8.** Spermatids; scale = 20 μm ; **9.** Spermatozoa; scale = 10 μm ; **10.** Condensed chromatin of spermatids and spermatocytes during postmoult; scale = 100 μm . Legend: s, spermatocytes; sp, spermatids.

matozoa constitute about 22% and 19%, respectively (Table 2).

Premoult: Testes from premoult males are characterized by a significant rise ($t = 3.205$; $p < 0.0125$) in proliferative activity (22%) from intermoult levels (16%); the F -test ($F = 1.18$) suggests that the difference between the 2 SDs is not significant ($p > 0.44$). The percentage of spermatids was remarkably low ($F = 105.48$; $p < 0.0001$) (Table 2).

Postmoult: Gonial proliferation tends to be at a low rate in postmoult crabs (within 24 h after ecdysis). Intense chromatin condensation appears to be a characteristic feature of the spermatocytes (57%) of postmoult crabs, while spermatids appear pycnotic (Fig. 10).

DISCUSSION

The events in setal development of the maxilliped epipodites described in this paper provide useful information on the cuticular and epidermal changes in relation to the moult cycle. This in turn, will help define the moult stages precisely, without unduly harming the animal. In order to identify the moult stages, the technique described in the paper can be applicable equally in both sexes of the brachyuran crab. The moult stages of *M. messor* appear to be comparable to the pattern exhibited by the field crab, *Paratelphusa hydrodromous* (Anilkumar 1980), while it differs from that of *Palaeomonetes pugio* (Freeman and Bartell 1975) which has a prolonged premoult stage. In the majid crab,

Chionoecetes opilio, the premoult has a longer duration, and stage D_1 is divided into substages such as D_1' , D_1'' , and D_1''' (Moriyasu and Mallet 1986, O' Halloran and O' Dor 1988). Such subdivisions do not seem to be pertinent in *M. messor* (present study) which has a shorter duration (3 d) of premoult.

The entire testicular mass of *M. messor* is tubular and comparable to the structure in *Chionoecetes opilio* (Kaestner 1970, Kon and Honma 1970), but differs from that of the primitive brachyurans (Hartnoll 1979).

In paraffin sections, the stages of spermatogenesis in *M. messor* are distinguishable, and appear akin to those described in other brachyurans such as *Paratelphusa hydrodromous* (Gupta et al. 1989) and *Ranina ranina* (Minagawa et al. 1994). Our observations on the testicular acini of the intermoult crabs (Table 2) reveal that the proliferative and maturing phases of germ cells are more or less in uniform proportions during that stage. However, commencement of premoult resulted in reduction in maturation of germ cells, judged by a significant decrease ($p < 0.001$) in the proportion of spermatids (Table 2). Thus, it seems apparent that sperm maturation in *M. messor* is negatively related to the onset of premoult, which is also coupled with a marked rise in ecdysteroid levels (Table 1). It is not clear if the elevated ecdysteroid titre has a restraining influence on decapod spermatogenesis. However, an antagonism is suggested to exist between oocyte maturation and somatic growth in female brachyuran decapods (Anilkumar and Adiyodi 1985, Adiyodi 1988). Further, experiments conducted in our laboratory reveal that the ongoing vitellogenic activity in *M. messor* is arrested if the female enters premoult (Sudha and Anilkumar 1996). Such an antagonism found between oocyte maturation and somatic growth seems to be comparable with the (inverse) relation existing between sperm maturation and premoult, as borne out from our present study. Thus, we are tempted to suggest that a high ecdysteroid titre can exert an inhibitory influence on sperm maturation in *M. messor*. On the other hand, during the intermoult stage when normal spermatogenesis occurs, the serum shows the presence of 126.10 ± 23.76 ng (per 100 g body weight) ecdysteroids. This observation suggests that a low ecdysteroid titre does not restrain sperm maturation (Table 1). And it is not known whether this low ecdysteroid level is a requisite for normal spermatogenesis. Comparably, low levels of ecdysteroids are suggested to assist maturation (elongation) in the apyrene (anucleate) spermatozoa of the European corn borer *Ostrinia nubilatus* (Gelman et al. 1989).

Table 2. Spermatogenic activity in relation to different moult stages of *Metopograpsus messor* (values expressed in percentage) (mean \pm SD) (sample size in parantheses)

Developmental stage	Intermoult (5)	Premoult (5)	Postmoult (5)
Spermatogonia	15.8 \pm 2.7	21.5 \pm 2.9	Nil
Primary spermatocyte	23.7 \pm 3.6	38.3 \pm 7.5	56.6 \pm 6.3 ^a
Secondary spermatocyte	20.1 \pm 2.7	11.3 \pm 3.0	
Spermatids	21.5 \pm 2.3	8.3 \pm 1.5	37.2 \pm 4.7
$(F = 105.48)$ ($p > 0.0001$)			
Spermatozoa	18.7 \pm 2.4	22.3 \pm 4.2	6.5 \pm 1.8

^aDue to intense chromatin condensation, a distinction could not be made between primary and secondary spermatocytes. Spermatogonia in premoult $>$ intermoult ($t = 3.205$) ($p < 0.0125$). As per Tukey-Kramer multiple comparison test: spermatids in postmoult $>$ intermoult ($p < 0.001$); spermatids in postmoult $>$ premoult ($p < 0.001$); spermatids in intermoult $>$ premoult ($p < 0.001$).

A direct influence on spermiogenesis by factors regulating the moult cycle has been proposed in the crab *Libinia emarginata*, which exhibits terminal anecdyosis (Laufer et al. 1993). Barring this observation, the exact influence, if any, of ecdysteroids in brachyuran spermatogenesis, is not clear. However, the role of ecdysteroids in enhancing spermatogenic activity has been well documented in insects (Hoffmann et al. 1980, Gelman et al. 1989, Raabe 1989, Becker et al. 1991, Gu and Chow 1996). A sex-related differences in haemolymph ecdysteroid levels during the postembryonic stage has been found in the hevea tussock moth, *Orgyia postica*. Such a dimorphic titre is suggested to prolong the feeding period in females so as to ensure maximum egg production (Gu et al. 1992). A paucity of literature does not permit us to draw comparisons with other brachyuran species. However, studies conducted on some non-brachyuran decapods are worth recalling in this context. For instance, the significantly high level of spermatogonial proliferation ($p < 0.0125$) in *M. messor* during premoult, i.e., under high ecdysteroid titre (240.70 ± 35.15 ng/100 g body weight) (Tables 1, 2), seems concordant with the increased spermatogonial proliferation shown by premoult males of the prawn *Macrobrachium idella* (Sreekumar and Adiyodi 1983) and lobster testis when exposed to high ecdysteroid concentrations (Brody and Chang 1989). A direct stimulatory effect of 20-hydroxyecdysone on testicular ^3H thymidine incorporation has been demonstrated in the natantian prawn *Macrobrachium rosenbergii* through in vitro experiments (Sagi et al. 1991).

We are unable to suggest any reason for the high degree of chromatin condensation shown by the spermatocytes and spermatids of postmoult crabs, although a comparable situation was found in *Paratelphusa hydrodromous* (Gupta et al. 1989).

To sum up, the results of the present study strongly indicate that, in *M. messor*, the elevated ecdysteroid titre coupled with the onset of proecdysis can enhance spermatogonial proliferation, but, on the other hand, it can restrain sperm maturation. Further, normal spermatogenesis, as seen in the intermoult stage, takes place under a low ecdysteroid titre. Whether a low ecdysteroid titre is a requisite for normal spermatogenesis to occur is a question that warrants more research.

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蟹類 *Metopograpsus messor* (Brachyura: Decapoda) 之蛻皮類固醇 激素濃度與精子形成相互關係

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本研究探討雄性平分大額蟹 (*Metopograpsus messor*) 生長與生殖之相互關係。不同蛻皮階段是以上肢顎足的剛毛發育而區分。在蛻皮間期，*M. messor* 的精巢，呈現各階段之精子形成（例如精組細胞、精母細胞、精子細胞及精子），且其比例亦相近。但在蛻皮前期開始（蛻皮類固醇激素濃度上升），引發精母細胞增殖，及精子成熟遲緩。雖然本研究結果指出在蛻皮間期，蛻皮類固醇激素分泌甚低，但精子形成仍然進行。

關鍵詞：甲殼類，上肢，生長，生殖，精子。

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