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Change in epidermal commitment and its hormonal control in *Pieris rapae*[1]

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Shi-Hong Gu, Yien-Shing Chow and Fei-Jann Lin (1993) Change in epidermal commitment and its hormonal control in *Pieris rapae*. *Bull. Inst. Zool., Academia Sinica* **32**(1): 23-31. To determine changes in the commitment of epidermal cells from larval to pupal development in *Pieris rapae* during the last larval instar, characteristics of the newly-secreted cuticles were examined and assessed via a scoring system after injections with 20-hydroxyecdysone. Pupal commitment in different epidermal regions occurred at different times; for example, the antenna and eye regions were committed to pupal development at the last larval ecdysis, but the regions along the mandible and thoracic legs were committed last. The fact that the inhibitory effect of juvenile hormone mimic on the pupal commitment was observed in a dose-dependent manner supports the idea that the pupal commitment of different epidermal regions might occur at different threshold levels of juvenile hormone. Starvation and the injection of small doses of 20-hydroxyecdysone also delayed the developmental programme. Hemolymph ecdysteroid titers were maintained at very low levels (0-6.8 ng/ml) during the feeding period of the last instar until just before wandering stage, and then increased sharply to a major peak in pharate pupae.

Key words: *Pieris rapae*, Commitment of epidermal cell, Ecdysteroid titer, Juvenile hormone mimic.

Larval-pupal metamorphosis in holometabolous insects occurs at the end of the last larval stage and is coordinated by hormones. However, prior to metamorphosis the epidermal cells acquire a new developmental commitment when juvenile hormone titer declines to a threshold level during a critial period (Riddiford 1976, 1978, 1985). This change in commitment has been observed to be coincident with a small peak of ecdysteroid titer in many species of insects (Lafont *et al.* 1977; Bollenbacher *et al.* 1981).

The expression of the ability to secrete pupal cuticle normally occurs at the end of the last larval instar, at which time the molting hormone (20-hydroxyecdysone) titer increases to a major peak. But when the ecdysteroid level is artificially elevated via injections with 20-hydroxyecdysone prior to the occurrence of the natural peak, the commitment can be expressed earlier in the last larval instar (Nijhout 1976; Hwang-Hsu *et al.* 1979; Calvez 1981; Yin and Chaw 1984). Changes in the pupal commitment of epidermal cells during the last larval instar have been demonstrated in many species of insects (Nijhout 1976; Hwang-Hsu *et al.* 1979; Calvez

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1981; Connat *et al.* 1984; Yin and Chaw 1984; Kremen and Nijhout 1989).

Pupal commitment in different epidermal regions occurs at different times (Yin and Chaw 1984). Differences in the sensitivity of tissues to the hormones that control metamorphosis are believed to be involved in the complex temporal and spatial sequence of pupal commitment (Fukuda 1952; Truman *et al.* 1974; Ohtaki *et al.* 1986). For example, the different epidermal regions of *Manduca sexta* require different durations of exposure to ecdysteroid to become committed *in vivo* (Truman *et al.* 1974) and *in vitro* (Mitsui and Riddiford 1978). Tissue-specific differences in the required duration of juvenile hormone-free time for pupal commitment have also been observed in *Bombyx mori* (Fukuda 1952; Ohtaki *et al.* 1986). In *Precis coenia*, evidence suggests that an intercellular communication signal is also involved in the regulation of pupal commitment (Kremen 1989).

The present study examines changes in the commitment of epidermal cells and their relationship with juvenile hormone and ecdysteroid levels during the last larval instar of *Pieris rapae*. The effects of starvation and injections of small doses of 20-hydroxyecdysone on pupal commitment are also discussed.

MATERIALS AND METHODS

Experimental animals

Pieris rapae crucivora larvae were reared on cabbage leaves at 24 ± 1°C under a long photoperiod (16L:8D). Larvae exhibiting head-capsule slippage to the fifth instar were selected from a mass-rearing container and put into a container without food. Newly-ecdysed larvae were grouped and used for experiments. Each group consisted of 20 larvae.

Application of hormones

A juvenile hormone mimic, hydroprene (Zoecon Corp., CA), was dissolved in acetone (HPLC grade) at various concentrations; 5 μl of solution was topically applied near the dorsal midline of the larval abdomens. Control specimens were treated with 5 μl of acetone only.

20-hydroxyecdysone (Sigma, St louis, MO, U.S.A.) was dissolved in 10% ethanol; its concentration was checked spectrophotometrically at 240 nm (Meltzer 1971). After preliminary testing of different doses, we decided to give each larva a single injection of 10 μg of 20-hydroxyecdysone. Two injections of each lower doses (5 μg) of 20-hydroxyecdysone were given 12 h apart; these lower doses had the same effect on pupal commitment. Larvae apolysed and deposited a new cuticle within two days after injection. To examine the effects of small doses of 20-hydroxyecdysone or starvation on pupal commitment, newly-ecdysed last-instar larvae were injected with 0.5 μg of 20-hydroxyecdysone per insect, or starved for 24 h, then fed under normal conditions. At 60 h, these larvae were injected with 10 μg/larva of 20-hydroxyecdysone.

Estimation of the developmental status of epidermal cells

The developmental status of epidermal cells was estimated according to the morphology of the newly-secreted cuticle as induced by exogenous 20-hydroxyecdysone. The graded morphological changes from larva to pupa were scored according to parameters listed in Table 1.

Radioimmunoassay of hemolymph ecdysteroid titer

Hemolymph (10 to 100 μl from each animal) was collected from the prolegs of

Table 1. Scoring system for pupal commitment following an injection of 20-hydroxyecdysone (10μg/larva)

score	characteristics
0	normal larval cuticle
1	larval cuticle, but with swollen antennae, partially evaginated wing discs, and a few discrete patches on pupal cuticle in dorsal prothorax.
2	approximately 75% larval cuticle and 25% pupal cuticle.
3	approximately 25% larval cuticle and 75% pupal cuticle.
4	normal pupal cuticle with some abnormality in the head.

precisely-timed larvae and stored at –70°C before use. Hemolymph samples were mixed with methanol, then centrifuged at 10,000 rpm for 5 min. The supernatant was evaporated to dryness and subjected to radioimmunoassay (Takeda *et al.* 1986; Gu *et al.* 1992a, b)

RESULTS

Switchover in the commitment of the epidermal cells defined by 20-hydroxyecdysone

The switchover in the epidermal commitment from larva to pupa was determined by injections with high doses of 20-hydroxyecdysone (10 μg per animal) and examinations of the type of cuticle secreted. During the first 24 h after the last larval ecdysis, secreted cuticle defined by 20-hydroxyecdysone showed almost all larval characteristics; however, pupal-like cuticle along eye regions (Fig. 1b), elongated and swollen antennae, and partially evaginated wing discs were also observed (Figs. 1b and 2b). In a few cases, patches of pupal cuticle in the dorsal-prothoracic region were observed (results not shown).

Injections administered at later times resulted in secreted cuticle with characteristics of larval-pupal intermediates. For instance, cuticle secreted after an injection at 36 h showed some pupal cuticle in both the dorsal-prothoracic regions and regions along the dorsal vessel (Fig. 2c). Injections at 48 h resulted in the appearance of more pupal cuticle, not only in the prothoracic and caudal segments, but also in the abdominal segments (Fig. 2d). At 60 h, injections resulted in the formation of pupae with patches of larval cuticle in the abdominal, mandibular, and thoracic leg regions (Fig. 2e).

To quantify the pupal commitment of epidermal cells defined by injections of 20-hydroxyecdysone at different times, the percentage of pupal cuticle was approximated and scored according to the parameters shown in Table 1 (Fig. 3). The percentage of pupae which responded to injections of 20-hydroxyecdysone by forming larval-pupal intermediates increased with age of injection. Some small patches of pupal cuticle appeared first in the dorsal-prothoracic region and in the regions along the dorsal vessel; such patches expanded as larvae continued their development. When pupal cuticle appeared in some abdominal regions,

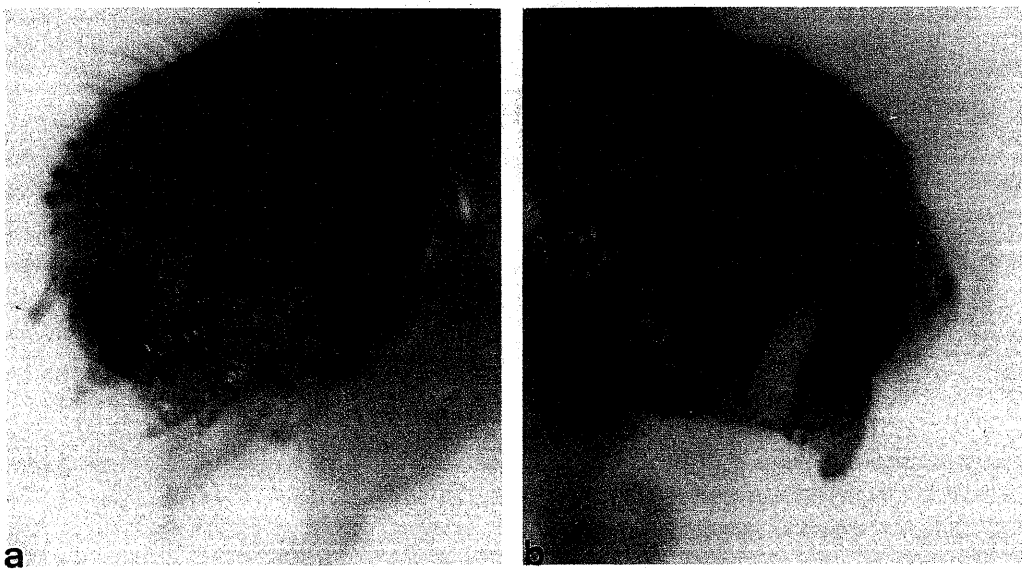

Fig. 1. The head of a normal last-instar larva (a) and an induced abnormal larva after 20-hydroxyecdysone injection during the first 24 h of the last instar (b). Arrows indicate elongated and swollen antenna and abnormal cuticle (larval-pupal intermediate) along the eye regions.

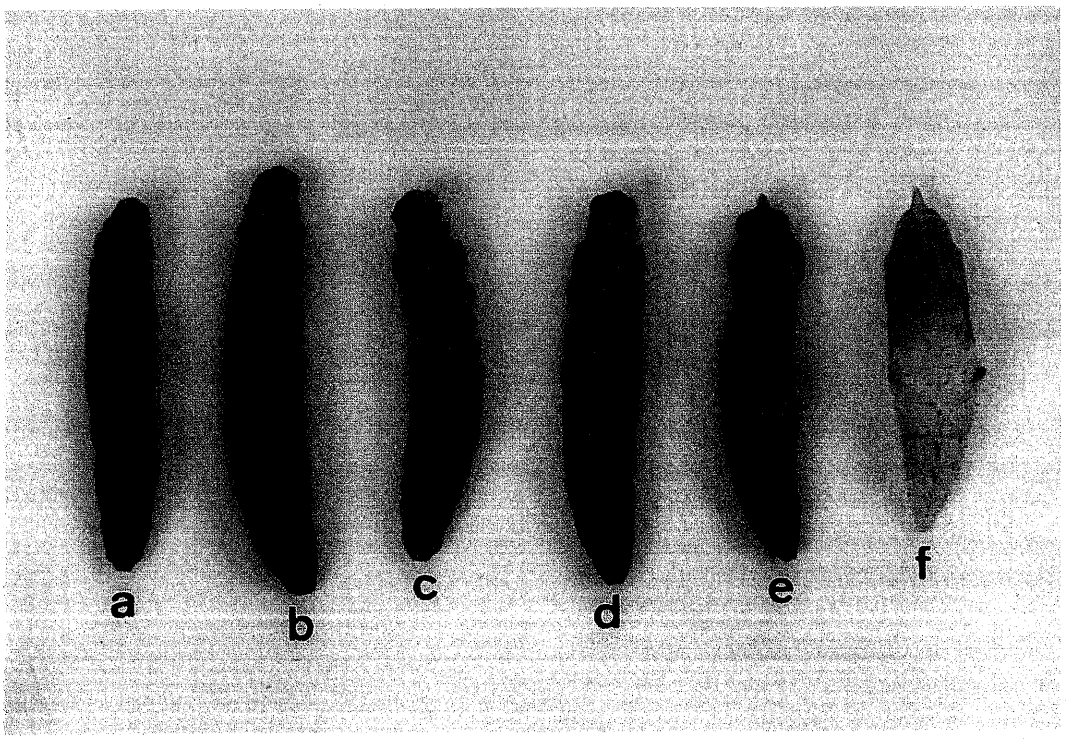

Fig. 2. Various types of larval-pupal intermediates produced after injections of 10 μg/larva of 20-hydroxyecdysone at different times during the last larval instar. (a) normal last-instar larva; (b-e) intermediates produced by injections of 20-hydroxyecdysone at 24 h (b), 36 h (c), 48 h (d), and 60 h (e); (f) normal pupa.

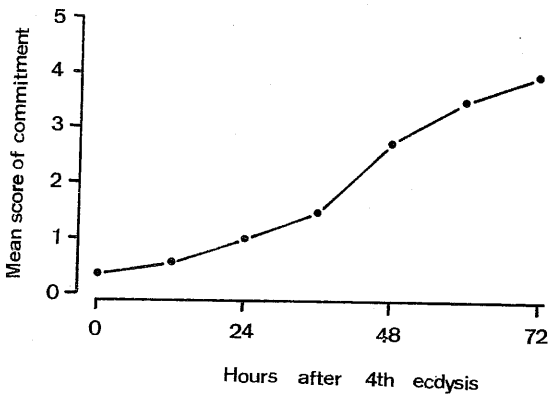

Fig. 3. Effect of 20-hydroxyecdysone (10 μg/larva) on pupal commitment. Larvae were injected with 20-hydroxyecdysone at different times during the last larval instar. Following injections, newly-secreted cuticle was evaluated using a 0 to 4 scoring system (Table 1). Data are based on the mean score obtained by a group of larvae injected at the same stage ($N=20$).

Table 2. Effect of topical application of hydroprene on pupal commitment

Dosage (μg)	mean score *
10	0.4 ± 0.1
1	1.1 ± 0.2
0.1	2.5 ± 0.1
0.01	2.9 ± 0.3
0	3.4 ± 0.2

Newly-ecdysed last-instar larvae were topically treated with different doses of hydroprene, then fed under normal conditions. At 60 h, the treated larvae and control larvae (treated with acetone) were injected with 10 μg/larva of 20-hydroxyecdysone. After injection, newly-secreted cuticle was examined and scored according to Table 1. *$N=20$.

adjacent patches of pupal cuticle in the dorsal prothoracic regions and the regions along the dorsal vessel merged. The regions along the mandible and thoracic legs were committed last.

Effect of application of juvenile hormone mimic on pupal commitment

Newly-ecdysed last-instar larvae were pre-treated with different doses of juvenile hormone mimic (hydroprene; 0.01-10 μg per animal), then fed under normal conditions. At 60 h, the larvae were injected with 10 μg of 20-hydroxyecdysone. After injection, the newly-secreted cuticle was examined and assessed according to the scoring system outlined in Table 1. As shown in Table 2, the topical application of hydroprene clearly inhibited the appearance of pupal characteristics. The higher the dosage used, the more larval cuticle was secreted. Score values of larvae injected with high doses of 20-hydroxyecdysone at 0 h (Fig. 3) and from larvae pre-treated with 10 μg of hydroprene at 0 h and injected with 20-hydroxyecdysone at 60 h show that this dosage of hydroprene completely inhibits the pupal commitment of the epidermis. However, the pre-treatment with 0.01 μg of hydroprene partially delayed pupal commitment.

Effect of small doses of 20-hydroxyecdysone on pupal commitment

While larvae apolysed and deposited new cuticle following injections with high doses of 20-hydroxyecdysone (10 μg per animal), a lower dosage (0.5 μg per animal) did not have the same effect. Therefore, we examined the effect of small doses of 20-hydroxyecdysone on pupal commitment.

Newly-ecdysed last-instar larvae were injected with small doses of 20-hydroxyecdysone (0.5 μg per animal), then fed under normal conditions. At 60 h, these larvae were injected with 10 μg/larva of 20-hydroxyecdysone. The newly-secreted cuticle was then examined and scored. The inhibitory effect of small doses of 20-hydroxyecdysone on pupal commitment was observed as compared with the control larvae, as shown in Table 3.

Table 3. Effect of starvation and small doses of 20-hydroxyecdysone on pupal commitment

treatment	mean score *
starvation	1.5 ± 0.2
20-hydroxyecdysone injection	2.0 ± 0.1
control	3.4 ± 0.2

Newly-ecdysed last-instar larvae were starved for 24 h or injected with 0.5 μg of 20-hydroxyecdysone per animal, then fed under normal conditions. Larvae injected with distilled water or non-starved larvae were used as control larvae. At 60 h, both treated and control larvae were injected with 10 μg/larva of 20-hydroxyecdysone. After injeciton, the resulting cuticle was examined and scored according to Table 1. *N = 20.

Effect of starvation on pupal commitment

Newly-ecdysed last-instar larvae were starved for 24 h, then fed under normal conditions. At 60 h, these larvae were injected with 10 μg/larva of 20-hydroxyecdysone; secreted cuticle was then examined. As shown in Table 3, there was definitely a delay of pupal commitment in the starved larvae when compared with the control larvae.

Ecdysteroid titers during the last larval instar

Changes in the ecdysteroid levels of hemolymph during the last larval instar are shown in Fig. 4. Hemolymph ecdysteroid titer remained low during the feeding period of the last larval instar (0-6.8 ng/ml). It then increased sharply just before the wandering stage and reached a high peak (204 ng/ml) at the pharate pupal stage. Titer then declined a few hours before larval-pupal ecdysis.

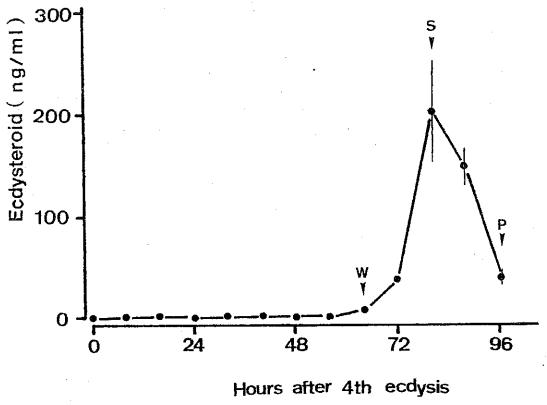

Fig. 4. Ecdysteroid levels in the hemolymph of last larval instar larvae of *Pieris rapae*. Each point represents an average value of 4 to 5 animals. Vertical lines are standard deviations. w: wandering; s: spinning; p: pupal ecdysis.

DISCUSSION

Our results clearly show that pupal commitment in different regions of epidermal cells during the last larval instar of *Pieris rapae* occurs in a specific time-dependent manner. Changes in commitment to metamorphosis first occur in the antenna and eye regions of the head as early as just after the last larval ecdysis; they then appear in the dorsal-prothoracic and dorsal-caudal regions, and finally are observable in the regions along the mandible and thoracic legs. Our observations of changes in the commitment of epidermal cells in *Pieris rapae* are consistent with those of Nijhout (1976) for *Manduca sexta*, Hwang-Hsu *et al.* (1979) for *Galleria mellonella*, Yin and Chaw (1984) for *Diatraea grandiosella*, Connat *et al.* (1984) for *Tenebrio molitor*, and Kremen and Nijhout (1989) for *Precis coenia*.

Changes in ecdysteroid titers during the last larval instar have been demonstrated in many species of insects (reviewed by Smith 1985). A small ecdysteroid peak, which occurs at the end of the feeding period just before the major ecdysteroid peak may be

responsible for changes in epidermal commitment (Truman *et al.* 1974; Riddiford 1976, 1978, 1985). However, in *Galleria* (Hwang-Hsu *et al.* 1979) and *Bombyx* (Calvez 1981) epidermal cells could express pupal characteristics at a stage prior to the appearance of the first (i.e., smaller) ecdysteroid peak. Our results clearly show that ecdysteroid titers during the last larval instar of *Pieris rapae* were always maintained at very low levels (0-6.8 ng/ml) until just before the wandering stage. After the wandering stage, ecdysteroid titers increased sharply to a major peak of 204 ng/ml in pharate pupae. Last-instar larvae could be defined to secrete cuticle with larval-pupal or pupal characteristics by injecting them with 20-hydroxyecdysone at various stages when hemolymph ecdysteroid titer levels were low (0-6.8 ng/ml, Fig. 4). Thus, our results correspond well with those of Hwang-Hsu *et al.* (1979) and Calvez (1981), and also suggest that ecdysteroids may play a secondary role in changes of epidermal commitment. In addition, observed changes in ecdysteroid titer levels during the last larval instar of *Pieris rapae* were different from those reported for *Pieris brassicae*, in which two successive peaks were observed (Lafont *et al.* 1977).

According to previous research, the switchover from larval to pupal commitment is controlled by juvenile hormone. When juvenile hormone levels are high, the genetic reprogramming of epidermal cells is inhibited, and the larval gene set remains active (Riddiford 1976, 1978, 1985). However, when juvenile hormone levels decline to a sub-threshold level, the epidermal cells change their commitment from larva to pupa in a temporal and spatial sequence (Kremen and Nijhout 1989). The inhibitory effect of juvenile hormone on pupal commitment was also observed in our experiments. When larvae, treated with different doses of hydroprene (0.01-10 μg per animal) just after the last larval ecdysis were injected with 20-hydroxyecdysone at 60 h, the resulting cuticle showed some juvenile characteristics as compared to control larvae (Table 2). Further, higher doses of juvenile hormone mimic resulted in more juvenile characteristics. The pupal commitment of different tissues in *Bombyx mori* occurs in a specific time-dependent manner in the absence of juvenile hormone (Fukuda 1952; Ohtaki *et al.* 1986). In the present study, different degrees of sensitivity to juvenile hormone mimic at different epidermal regions suggest that different epidermal regions may change their commitment at different sub-threshold levels of juvenile hormone. Our results show that the antenna and eye regions may change their commitment at a higher sub-threshold level of juvenile hormone as compared to those regions along the mandible and thoracic legs, which may require a much lower sub-threshold level. In *Manduca sexta*, different epidermal regions have been found to require different lengths of exposure to prothoracic gland secretions in order to form pupal cuticle (Truman *et al.* 1974); such a tissue-specific ecdysteroid requirement for pupal commitment has also been observed *in vitro* (Mitsui and Riddiford 1976). Similar *in vitro* research regarding *Pieris rapae* will be conducted in the future.

When larvae were starved for 24 h or injected with small doses of 20-hydroxyecdysone during the early stages of the last larval instar, pupal commitment was delayed as compared with control larvae. Similar effects of starvation have been demonstrated in *Calliphora* and *Sarcophaga* (Zdarek and Slama 1972). Evidence suggests that during the starvation period, a decline in juvenile hormone levels and a rise in juvenile hormone esterase levels are prevented (Reddy *et al.* 1979; Cymborowski *et al.* 1982). On the other hand, injections of small doses of 20-hydroxyecdysone during the early stages of the last larval instar of *Calliphora* and *Sarcophaga* were shown to activate the pupal programme (Zdarek and Slama 1972). These

conflicting effects of small doses of 20-hydroxyecdysone may simply reflect a difference between species, or they may be the result of different concentrations of the hormone and/or differences in the developmental stages of the animals (Kubo *et al*. 1983). When last-instar larvae of *Bombyx mori* receive small doses of 20-hydroxyecdysone via diet during their early stages, supernumerary larval molts are induced; it has been suggested that 20-hydroxyecdysone stimulates the secretory activity of the corpora allata, which normally would stop secreting juvenile hormone (Gu unpublished). Thus, further study is needed to determine whether levels of ecdysteroid and/or starvation during the early stages of the last larval instar alter the secretion of juvenile hormone.

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紋白蝶表皮組織發育程序之變化及激素控制

顧 世 紅　周 延 鑫　林 飛 棧

本實驗利用注射脫皮激素後新形成之表皮的形態，推斷了最後一齡紋白蝶幼蟲的表皮細胞從幼蟲至蛹之發育程序的變化。結果表明：不同區域之表皮組織的發育程序在不同的時間內發生變化，例如，觸角及單眼之區域的發育程序之變化發生在最後一齡幼蟲脫皮時，但大顎及胸腳是最後發生變化的。青春激素類似物對表皮組織的發育程序之變化具有抑制作用，且與其劑量有關，這一事實表明，不同區域之表皮組織也許在體內不同的青春激素臨界值下發生從幼蟲至蛹的發育程序之轉變。五齡初期的絕食及少量的脫皮激素的注射對發育程序之變化也具有抑制作用。血液中脫皮激素濃度變化表明，最後一齡攝食期其濃度很低（0-6.8 ng/ml），進入 Wandering 期後增加，在化蛹前達到高峰。

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Microencapsulated LH-RH analog accelerated spermiation in protandrous black porgy, *Acanthopagrus schlegeli*

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The objective of this study was to investigate the regulation of reproduction and gonadal steroids in two-year-old protandrous black porgy through treatment with microencapsulated D-Trp^6-luteinizing hormone-releasing hormone (LH-RH analog). Sixteen black porgy were equally divided into two groups and injected with saline and microencapsulated LH-RH analog, respectively, Spermiation and plasma levels of testosterone (T), estradiol-17β (E_2), and 17α-hydroxyprogesterone (17α-OH P) were measured just before and after treatment at intervals of 1-2 weeks for four months. Microencapsulated LH-RH analog accelerated the onset of spermiation by three weeks. Percentages of spermiating fish were higher in the microencapsulated analog group (100%) than the control group (50%). During the experimental period the total amount of milt produced was higher in the microencapsulated analog group (84 ml) than in the control group (7 ml). Peak levels of plasma T occurred before the onset of spermiation in both the LH-RH analog group and control group. Two other minor peaks of plasma T were also noted during the spawning season. Levels of plasma E_2 were higher in the control group as compared to the LH-RH analog group. Plasma 17α-OH P levels remained at undetectable levels in fish of both groups. Non-significant differences in gonadosomatic indices were observed; in addition, there were no sexual reversal fish in either group.

Key words: LH-RH analog, Microcapsule, Spermiation, Steroids, Black porgy

Superactive analogs of mammalian luteinizing hormone-releasing hormone (LH-RH) have been widely used to induce ovulation and spermiation in a number of teleosts, including: ayu, *Plecoglossus altivelis* (Aida, 1983; Hirose *et al.*, 1983); black porgy, *Acanthopagrus schlegeli* (Yueh *et al.*, 1990; Chang and Yuen 1990a; Chang *et al.*, 1991); walleye, *Stizostedion vitreum* (Pankhurst *et al.*, 1988); catfish, *Clarias macrocephalus* (Ngamvongchon *et al.*, 1986); carp (Ngamvongchon *et al.*, 1987); Atlantic salmon, *Salmo salar* (Weil and Crim, 1983; Crim and Glebe, 1984); seabass, *Lates calcarifer* (Harvey *et al.*, 1985); rabbitfish, *Siganus guttatus* (Harvey *et al.*, 1985); and milkfish, *Chanos chanos* (Lee *et al.*, 1986).

The black porgy is a marine protandrous hermaphrodite that is a valuable species in