Reference: *Biol. Bull.* **172:** 350–356. (June, 1987)

INDUCTION OF PRECOCIOUS MOLTING AND CLAW TRANSFORMATION IN ALPHEID SHRIMPS BY EXOGENOUS 20-HYDROXYECDYSONE

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ABSTRACT

Exogenous micromolar concentrations of 20-hydroxyecdysone were used to accelerate the molt cycle in the snapping shrimp, *Alpheus heterochelis*. Shrimps were exposed to the hormone by adding it to the seawater in which they were cultured. Five days of continuous exposure of an animal to a hormone concentration of 5 μgm/ml shortened the winter molt cycle length by 18 days, or 65%. Claw transformation, a commonly observed phenomenon in alpheid shrimps, also was accelerated at these concentrations, and postmolt animals exhibited appropriate modifications in external claw morphology and, in some cases, restructuring of the claw closer muscle according to schedules identical with those of animals having normal molt cycle lengths. These studies strengthen the hypothesis that the cellular events which underlie claw transformation are modulated by endogenous ecdysteroids.

INTRODUCTION

The insect growth hormone, 20-hydroxyecdysone, promotes molting in a variety of crustaceans, whether directly injected into subject animals (Krishnakumaran and Schneiderman, 1969, 1970; Freeman and Bartell, 1976; Rao *et al.*, 1973; Tighe-Ford and Vaile, 1972) or added to the medium of cultured larvae (Cheung, 1974). Normally during the crustacean molting cycle, alpha ecdysone is thought to be synthesized and released from the Y-organ, and then converted to 20-hydroxyecdysone in target tissues (Skinner, 1985). The widespread and dramatic effects of these steroids has promoted the acceptance of ecdysones as generalized arthropod growth hormones.

We were interested in determining whether exogenous 20-hydroxyecdysone would be effective in shortening the molt cycle and in accelerating claw transformation in the snapping shrimp, *Alpheus heterochelis*. These intertidal crustaceans have extremely asymmetric claws—a large snapper and a small pincer—that take part in a remarkable morphological transformation: If the large snapper claw is caused to undergo autotomy, the pincer claw transforms into a snapper, while a new pincer regenerates at the original snapper site (Przibram, 1901; Wilson, 1903). Claw transformation, however, involves more than growth and change in external morphology. The two muscles in the propodite—the opener and the closer—enlarge considerably during transformation. Their motor neurons also become proportionally larger (Mellon *et al.*, 1981; Mellon and Smith, unpub. obs.) and their physiological and biochemical properties are modified (Stephens and Mellon, 1979; Quigley and Mellon, 1984). Moreover, a dramatic instance of cellular death accompanies transformation of the claw. In the pincer the closer muscle is a composite of two different fiber types: There is a central band of large fast type fibers that is flanked on either side by smaller

Received 22 December 1986; accepted 17 March 1987.

diameter slower type fibers (Stephens and Mellon, 1979; O'Connor *et al.*, 1982). As the muscle transforms, the fast fibers die, while the slow fibers are modified to become very slow fibers (Quigley and Mellon, 1986). Muscle fiber death is apparent within a week following the first molt after onset of transformation; thus, both the change in claw morphology and the transformation of muscle fiber appear to be tied to the molt cycle, and both may be under hormonal control. To test this possibility, we exposed snapper-less shrimps to exogenous 20-hydroxyecdysone. The results show that the molt cycle can be reduced by as much as 65% in response to external ecdysone. Morphological claw transformation also was apparent in all of our experimental animals, while degeneration of the fast closer muscle fibers group occurred in two of the three experimental groups.

Materials and Methods

Experimental animals were recently molted, medium sized (4–5 cm) snapping shrimps (*Alpheus heterochelis*). Shrimps were housed individually in plastic containers in a 4 foot by 5 foot tray. Seawater entered the tray at one end, passed through the shrimp cages, and exited at the other end of the tray. Within a day of molting, animals were removed from the culture system and isolated individually in plastic containers with 100 ml artificial seawater (Forty Fathoms, Marine Enterprises Inc., Towson, Maryland).

The animals were exposed to exogenous hormone by adding to an individual animal's plastic container 100 microliters of a stock solution containing either 0.05 mg/ml or 0.5 mg/ml of 20-hydroxyecdysone (Sigma, E-2003). The stock solution was originally prepared by dissolving the hormone in 5% ethanol to the desired concentration.

Three different experimental protocols were used in our experiments. In the first protocol, shrimps were taken on the day following a molt, caused to autotomize the snapper claw, and placed immediately in the hormone bath. Hormone exposure was limited to five days, following which the animals were put into plain artificial seawater.

The second protocol involved removal of the snapper on the day following a molt and then waiting five days before exposure to hormone. In the third protocol, animals were left undisturbed for six days after molting at which time their snapper claws were removed. The animals were then placed in the hormone bath.

Regardless of the protocol, the animals were fed on the tropical fish food Tetramin every other day after which the bath water was changed. Molt cycles of animals exposed to the hormone were compared with those of animals kept in our culture system and from which the snapper claw had been removed two days following a molt. These control animals were obtained throughout the year, which was arbitrarily divided into winter (October–March) and summer (April–September). Our experiments were performed during February and March or during October and November. Data from the two groups were pooled. Room temperatures during the experimental periods varied between 22° and 24°C. Room lighting was diurnally supplemented through large glass windows. Seasonal variations in day length thus may have been sensed by the experimental animals.

The molt cycle of crustaceans has been divided into stages by various workers (Skinner, 1985). These differ qualitatively even across different groups of decapods. While there may be a correlation with the various molt cycle stages and the level of ecydsone in the hemolymph, this has not been measured in any shrimp species. In the crayfish *Orconectes,* however, both *in vivo* and *in vitro* measurements of ecdysone production suggest that the hemolymph titer begins to rise during the early stages of

premolt and reaches a peak in late premolt (Jegla *et al.*, 1983). The blood hormone level then falls to low levels just prior to ecdysis.

We measured the molt cycle lengths in 36 snapper-less summer shrimps, 28 snapper-less winter shrimps, and in 43 ecdysone-treated snapper-less winter animals. Molt cycle length was taken as the number of days between two successive ecdyses.

Immunohistochemical procedures for examining biochemical and structural changes in the closer muscles of transforming claws have been described previously (Quigley and Mellon, 1986). Briefly, transforming claws were removed from animals eight days following the first molt after snapper claw autotomy. The claws were immediately frozen in melting isopentane and sectioned on a cryostat at 30 micrometers thickness. Frozen sections were mounted on glass coverslips and either stored at −80°C, or they were immediately reacted with monoclonal antibodies developed in our laboratory against fast and slow shrimp muscle myosin heavy chains. Muscle degeneration was assessed by microscopic examination of the stained, antibody-reacted sections. Fast fiber histolysis was presumed to have occurred when the fast-specific antibody reaction could no longer be detected in the central regions of the closer muscle. Usually, the absence of fast muscle fibers was also indicated by a tissue-free space in the central muscle. In any case, the reaction of adjacent frozen sections with antibody to slow fiber myosin assured that the tissues and reaction mixtures were normal.

RESULTS

Figure 1 illustrates various stages in the morphological transformation of pincer to snapper. Not all stages are observed in any one animal during transformation. The earliest obvious morphological clue that transformation of the claw is occurring is the appearance (after a molt) of a mesial ridge on the dactylus. This ridge represents the earliest expression of the plunger on the fully transformed dactyl. We used the occurrence of any of the stages in Figure 1 as an indication that morphological transformation of the claw was occurring.

Our data, shown in Figure 2, compare molt cycle lengths in snapper-less summer and winter shrimps, and in ecdysone-treated snapper-less winter animals. On average, winter (October–March) molt cycles in transforming shrimps lasted about 29 days, while transforming summer (April–September) animals molted about every 23 days. This finding is similar to other studies of seasonal molt cycle change (*e.g.*, Freeman and Bartell, 1975). The underlying physiological reasons for the longer length of molt cycle in winter animals are not known; room temperatures in general were about two degrees higher in the summer, but other factors, such as day length, may play a significant role in determining growth and molting.

It is clear that treatment with 20-hydroxyecdysone at the concentrations we employed significantly shortened the usual winter molt cycle. Treatment in the experimental groups of animals was started either in February or early October, and experiments continued for two months. Normal, snapper-less animals from this time period would have had a molt cycle length of about 29 ± 3.8 days; those treated for five days with 20-hydroxyecdysone at the lower concentration we employed had a molt cycle of about 21 ± 4.8 days, while at the higher concentration the effect was more dramatic and the molt cycle length was only 10.5 ± 1.5 days.

Three different experimental protocols were used in exposing the experimental animals to the higher concentration of exogenous hormone. Figure 3 is a diagram illustrating these application schedules and the consequent effects upon a "normal" molt cycle length of 29 days. Shrimps undergoing protocol #1 molted, on average, 10.5 days after their previous molt, thus shortening their normal intermolt interval by 65%.

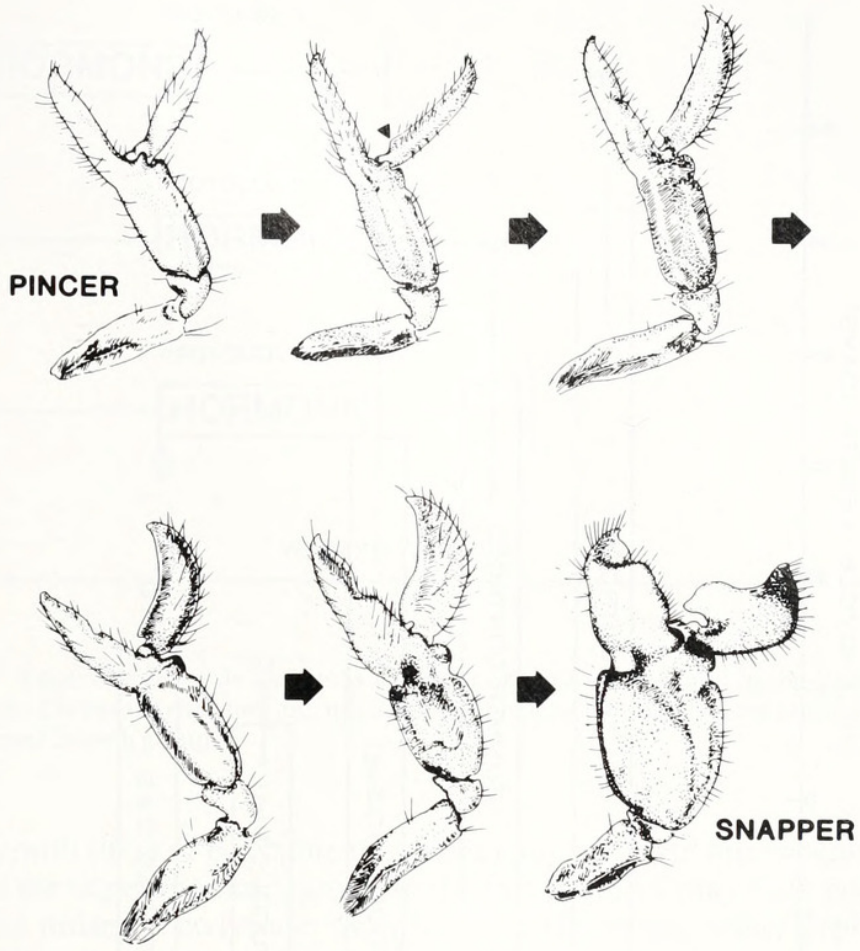

FIGURE 1. Morphological stages in the transformation of a pincer claw to a snapper claw. As a rule no single animal successively exhibits all of the stages illustrated, and in some instances, the stage exhibited at the first molt during transformation is considerably advanced. The caret indicates the earliest stage observed in the formation of the plunger found on the dactyl of the fully formed snapper.

Shrimps in the other two protocols molted roughly 14 days following their previous ecdysis, shortening their intermolt interval by 52%. Thus, the maximum reduction in molt cycle length was obtained by beginning hormone treatment immediately following the previous ecdysis.

It was of interest whether hormone-induced precocious molting and the accompanying claw transformation would elicit the modifications in claw closer muscle structure that normally occur in snapper-less shrimps. Therefore the claws of all surviving, transforming experimental animals were subjected to immunohistochemical analysis, as described previously (Quigley and Mellon, 1986).

Table I indicates the incidence of muscle degeneration in each of the three experimental groups. While two of the groups exhibited a preponderance of muscle remodeling, the group in which hormone treatment was initiated directly after a molt gave no evidence of closer muscle degeneration even though, in every case, the external morphology of the claw was clearly changing.

DISCUSSION

A previous study (Freeman and Bartell, 1976) demonstrated that 20-hydroxyecdysone will shorten the molt cycle in another caridean shrimp, *Palaemonetes pugio.* Some differences are apparent between the results of that study and of our own exper-

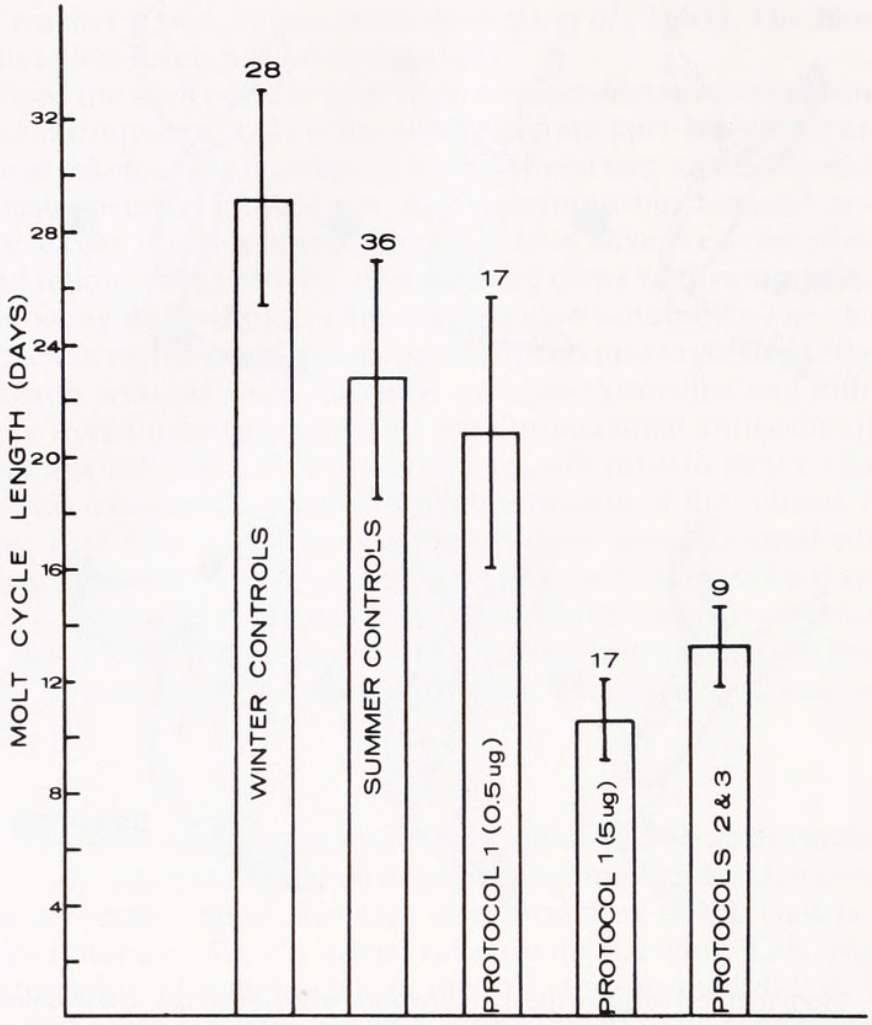

FIGURE 2. Bar graphs illustrating mean molt cycle duration in several groups of shrimps: Winter controls; summer controls; experimental animals in protocol 1 exposed to either 0.5 micrograms per milliliter or 5 micrograms per milliliter hormone concentration, and the pooled data from experimental animals in protocols 2 and 3, both of which were exposed to hormone concentrations of 5 micrograms per milliliter. Brackets at the top of each bar are ±one standard deviation, and numbers of animals in each group are shown above the brackets.

iments. In the study by Freeman and Bartell (1976), 20-hydroxyecdysone was injected into the experimental animals. Four different dosages were tried, and the lowest effective dosage (0.5 μgm/animal) shortened the intermolt cycle by 67%. Only 19% of this group survived the succeeding molt, however, whereas the overall survival rate in our three experimental groups was 72%. If the two shrimp species are physiologically comparable, it appears that simply bathing in the hormone is as effective a treatment as injection and is less traumatic to the experimental animals.

It is clear from our studies that exogenous 20-hydroxyecdysone accelerates not only the molt cycle in snapper-less *Alpheus,* but also the morphogenetic events that bring about transformation of the claw from a pincer to a snapper. This includes, in some cases, the programmed death of the fast group of muscle fibers in the claw closer. Fast fiber death was not seen in any of the experimental animals in protocol #1, in which hormone treatment was started one day following the preceding molt. The underlying physiological reasons for this failure are not known. In many decapod crustaceans the concentration of ecdysteroids in the hemolymph begins to rise in the premolt phase of the molting cycle, reaches a peak in mid premolt, and falls precipitously in late premolt, just prior to ecdysis (Jegla *et al.,* 1983; Skinner, 1985).

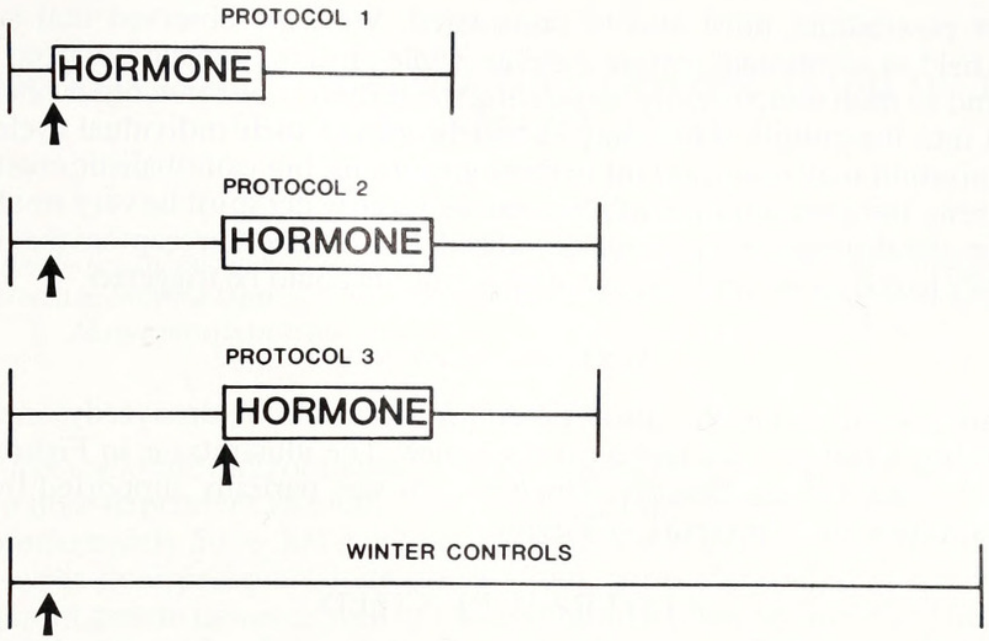

FIGURE 3. Relative molt cycle durations in winter control animals and in the three experimental protocols. Periods of hormone exposure are indicated, and arrows delineate the time point at which snapper claws were removed in each group.

If the hemolymph titers of ecdysone in *Alpheus* approximate this schedule, then it is apparent that the experimental animals in the first protocol may have been subjected to two distinct pulses of ecdysone, as opposed to the single, rather prolonged pulse which probably occurred in protocols #2 and #3. Since onset as well as reduction in blood hormone levels may act as a trophic signal (*e.g.*, Morton and Truman, 1985), the two pulses could have unpredictable consequences. Until the relative blood concentrations of ecdysone are precisely known throughout the intermolt period in *Alpheus,* however, further speculation cannot be justified. It is sufficient to conclude that, with an appropriate waiting period following molting, 5 days of treatment with exogenous ecdysone will shorten the mean normal molt cycle by more than 18 days, or by about 65%, and will proportionally accelerate the normal processes of claw transformation, including remodeling of the closer muscle. The procedure thus may prove to be useful as a tool in future experimental manipulations.

The mechanism of uptake (if any) of ecdysone from the bathing medium by the shrimps is not known. The gills of these intertidal shrimps are probably permeable to water and some inorganic ions, but it does not necessarily follow that molecules the size of steroids can pass into the blood through this route. Possibly, the hormone is ingested when the animals feed.

TABLE I

Effects of experimental hormone treatments on closer muscle restructuring in transforming claws

	Total number of animals	Number of successful molts*	Number of closer muscles examined	Number of restructured muscles
Protocol 1	30	27	10	0
Protocol 2	6	6	3	2
Protocol 3	14	5	3	2

* Attrition was due either to death of the animal or to autotomized pincer claws.

Other possibilities must also be considered. We have observed that snapping shrimps held in communal culture systems, while physically separated from one another, tend to molt concurrently, suggesting the ecdysone or some other pheromone excreted into the culture water may be synchronizing their individual cycles. Such synchronization may be important to these gregarious, but cannibalistic crustaceans. In any event, the concentration of pheromone in the water must be very small. If this is the case, the shrimps may possess specialized external chemoreceptors that respond specifically to ecdysone, and through which molting could be triggered.

ACKNOWLEDGMENTS

We are grateful to Dr. W. Otto Friesen for a gift of 20-hydroxyecdysone, and to W. D. Nelms Creekmur for technical assistance. The illustrations in Figure 1 were executed by Ms. Donna Bennett. This research was partially supported by an in-house grant from the University of Virginia.

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