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Authors: Yamamoto, Hisashi, Kawaii, Satoru, Yoshimura, Erina, Tachibana, Akiko, and Fusetani, Nobuhiro

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20-Hydroxyecdysone Regulates Larval Metamorphosis of the Barnacle, *Balanus amphitrite*

Hisashi Yamamoto[1]*, Satoru Kawaii, Erina Yoshimura,
Akiko Tachibana and Nobuhiro Fusetani

*Fusetani Biofouling Project, ERATO, Research Development Corporation of Japan,
c/o Niigata Engineering Co. Ltd., 27 Shin-isogo-cho, Isogo-ku, Yokohama 235, Japan*

ABSTRACT—The effects of 20-hydroxyecdysone (20-HE) on cyprid larval attachment and metamorphosis of the barnacle *Balanus amphitrite* were examined. When exposed to 10-100 μM 20-HE, cyprids failed to metamorphose, but attached to substrata. However, 0.001-0.1 μM 20-HE promoted attachment and metamorphosis. Moreover, effects of a combination of 20-HE and methyl farnesoate (MF) on cyprids were examined. The metamorphosis-inducing effect of MF was suppressed by pretreatment with 100 μM 20-HE. In contrast, after pretreatment with 10 μM MF, 100 μM 20-HE inhibited metamorphosis. High performance capillary electrophoresis (HPCE) revealed the presence of 20-HE in the extract from cyprid larvae of *B. amphitrite*, suggesting the intrinsic hormonal role of 20-HE in larval metamorphosis process.

INTRODUCTION

In sessile marine invertebrates, settlement competent larvae are thought to initiate attachment behavior upon reception of chemical cues. These cues are often derived from adult conspecifics, prey organisms, or biofilms (Morse, 1990). It has long been known that cyprid larvae respond to arthropodin, the protein found in adult barnacles (Larman *et al.*, 1982). When exposed to adult extracts, cyprids begin exploring potential attachment substrata and eventually attach by the secretion of cement, after which metamorphosis to the juvenile barnacle takes place. It is probable that chemoreception followed by signal transduction is involved in this transition.

In the barnacle *Balanus amphitrite*, we have already reported signal transduction involvement in larval attachment and metamorphosis of the barnacle *Balanus amphitrite*: 1) PKC signal transduction in metamorphosis (Yamamoto *et al.*, 1995); 2) serotonergic neurons involvement in settlement (Yamamoto *et al.*, 1996); 3) a hormonal substance, methyl farnesoate in metamorphosis via PKC activation (Yamamoto *et al.*, 1997a); 4) calmodulin participation in enzyme reactions in the process of larval attachment and metamorphosis (Yamamoto *et al.*, 1997b); 5) dopamine and serotonin involvement in larval attachment (Yamamoto *et al.*, 1997c). These suggest that larval attachment is controlled by neurotransmitters such as serotonin and dopamine, while larval metamorphosis is regulated by methyl farnesoate via PKC activation. In addition, calcium-calmodulin related enzyme reaction plays an important role in attachment and metamorphosis.

Cyprid larvae of barnacles were reported to be affected primarily by juvenile hormone, ecdysone and mimics of these substances: Farnesol was reported to promote cyprid and nauplius metamorphosis in a stage dependent manner in *Elminus modestus* (Mortlock *et al.*, 1984); the ecdysone mimic RH5849 promoted larval settlement and metamorphosis of *B. amphitrite* (Clare *et al.*, 1992); a juvenile hormone analog also induced metamorphosis of *E. modestus* (Tighe-Ford, 1977); juvenile hormone mimics (ZR-512, ZR-515) induced premature metamorphosis of *B. galeatus* (Gomez *et al.*, 1973); and 20-HE was found to be involved in the molt-cycle (Freeman and Costlow, 1983). In our previous study, we confirmed that MF and JH-III induce larval metamorphosis via PKC activation, however, only MF was detected in both adults and cyprid larvae of *B. amphitrite* (Yamamoto *et al.*, 1997a). In order to obtain further information on the function of hormonal substances in cyprid larval attachment and metamorphosis of *B. amphitrite*, effects of 20-HE on larval attachment and metamorphosis were examined and detection of 20-HE in cyprids was performed by high performance capillary electrophoresis.

MATERIALS AND METHODS

Materials

Artifical seawater (ASW) was prepared according to the Van't Hoff formula as follows: 460 mM NaCl, 10.1 mM KCl, 9.2 mM $CaCl_2$, 17.5 mM $MgSO_4$, and 10 mM Tris-HCl (pH 8.2). 20-hydroxyecdysone (20-HE) was purchased from Sigma Chemical Co. (St. Louis, USA). Methyl farnesoate (MF) was prepared from farnesoic acid, which was

* Corresponding author: Tel. +81-298-64-6712;
FAX. +81-298-64-6719.
[1] Present address: Maruha Corporation Central Research Institute, 16-2 wadai, tsukuba, Ibaraki 300-42, Japan.

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a generous gift from Dr. M. Wilder (Wilder *et al.*, 1995). γ-cyclodextrin was purchased from Wako Pure Chemicals Co. (Osaka, Japan).

Assays for larval attachment and metamorphosis

Competent cyprid larvae were obtained from laboratory cultured adult *Balanus amphitrite* using methods described previously (Yamamoto *et al.*, 1995). Assays for larval attachment and metamorphosis were performed using 6-well polystyrene plates (CORNING Cell Wells). Six milliliters of ASW was added to each well, plus 10 ± 2 cyprids. The plates were then placed on an orbital shaker and maintained at 22°C. Test solutions were prepared by addition of 6 μl of aqueous or dimethylsulfoxide (DMSO) solution of test samples to 6 ml of ASW. Plates were observed daily for a maximum of 6 days under a binocular dissection microscope. The number of larvae that metamorphosed and attached to substrata, metamorphosed and unattached, nonmetamorphosed and attached, and nonmetamorphosed and unattached was counted for each well. MF, 20-HE were dissolved in DMSO; DMSO alone showed no effects on larval attachment and metamorphosis at the concentrations used in this assay (Yamamoto *et al.*, 1995). Significant effects on larval attachment and metamorphosis were tested using analysis of variance (ANOVA), following which Tukey-Kramer multiple comparison tests (at $p<0.05$) were used to identify specific effects. Each drug treatment was replicated at least three times.

Microinjection of 20-HE into cyprid larvae

Larval movement was restrained by overnight storage at 4°C, following which cyprids were fixed loosely between two glass coverslips. 20-HE was dissolved in DMSO and injected into the inside of body between legs by a micromanipulator with a 7 μM diameter glass micropipette. The volume of 20-HE was determined according to the method previously reported (Yamamoto *et al.*, 1996).

High performance capillary electrophoresis

The detection of 20-HE were performed by the methods previously described (Yasuda *et al.*, 1993). Micellar solutions were prepared by dissolving 15 mM γ-cyclodextrin and 100 mM sodium dodecyl sulfate (SDS) in 100 mM borate buffer (pH 8.9). Standard 20-HE was dissolved in a micellar solution containing 50% (v/v) methanol, and injected by pressure of nitorgen gas for 20-60 seconds (a fused silica capillary, 75 μm × 50 cm, Beckman P/ACE 2100 system). Analysis was performed with an applied voltage of 25 kV at 20°C and with UV detection at 245 nm. Cyprids (approx. 5,000 individuals) were collected and then washed with ASW (2-3 times) and finally with distilled water (4°C). After washing, larvae were centrifuged at 4°C (2,000 × g, 10 min). The supernatant was removed and pellet steeped in 7 ml of acetone overnight at 4°C and then centrifuged. The supernatant was evaporated to dryness and patitioned between n-hexane (15 × 4 ml) and water (15 ml). The hexane soluble layers were combined and analyzed as mentioned above.

RESULTS

When cyprid larvae were exposed to 10 and 100 μM 20-HE, larvae attached to substrata, but failed to metamorphose (Fig. 1). At the beginning of these assays, most cyprids in ASW alone were swimming actively. Within one day, more than 30% of cyprids embarked on searching for suitable settlement site, attached to substrate, and metamorphosed into juveniles. On day 5, the percentages of attached and metamorphosed cyprids were increased to more than 60%. When exposed to high concentrations of 20-HE, nonmetamophosed, attached cyprids were observed at high frequencies. Statistical significance was as follows (ANOVA): day 1, juveniles (attachment and metamorphosis); $F=83.1742$, $p<0.0001$, nonmetamorphosed, attached cyprids; $F=287.5573$, $p<0.0001$. day 5, juveniles (attachment and metamorphosis); $F=46.5592$, $p<0.0001$, nonmetamorphosed, attached cyprids; $F=32.5391$, $p<0.0001$. The nonmetamorphosed, attached cyprids are shown in Fig. 2. In most of nonmetamorphosed, attached cyprids, apolysis occurred partially.

Lower concentrations of 20-HE (0.001-0.1 μM) significantly promoted larval metamorphosis (Figs. 1, 2). As described above, exposed to 10-100 μM 20-HE, cyprids attached to substrata but failed to metamorphose and molt into juve-

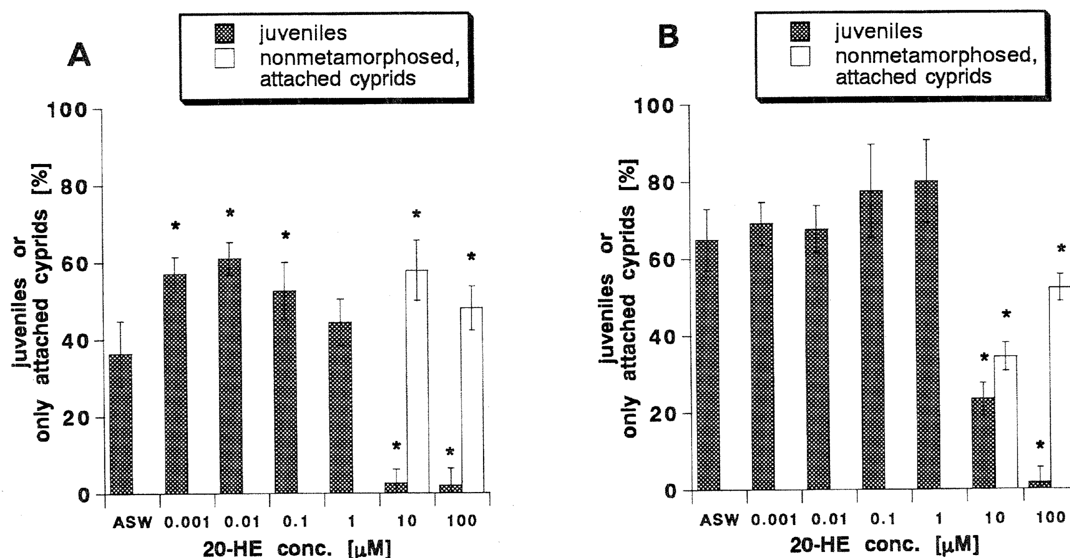

Fig. 1. Effects of 20-HE on cyprid larval attachment and metamorphosis. No unmetamorphosed cyprids were counted in controls, because no more settlement was occurring after 6 days. Data presented are means ± S.D. (**A**) day 1, (**B**) day 5. *: significant at $p<0.05$.

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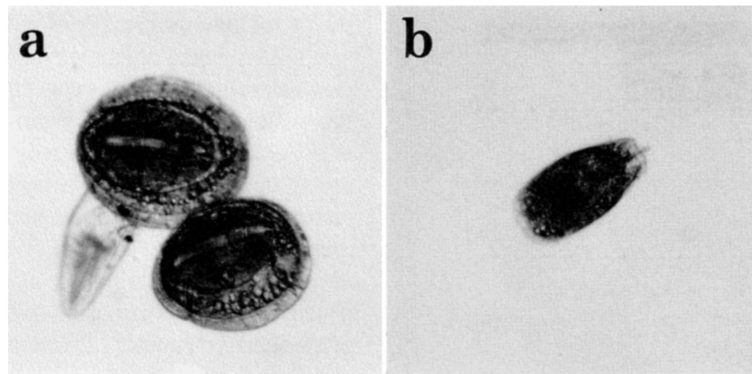

Fig. 2. Juvenile morphology affected by 100 μM 20-HE. (**a**) normal juveniles (attached and metamorphosed), (**b**) nonmetamorphosed, attached cyprids by 100 μM 20-HE effect. Scale bar; 300 μm.

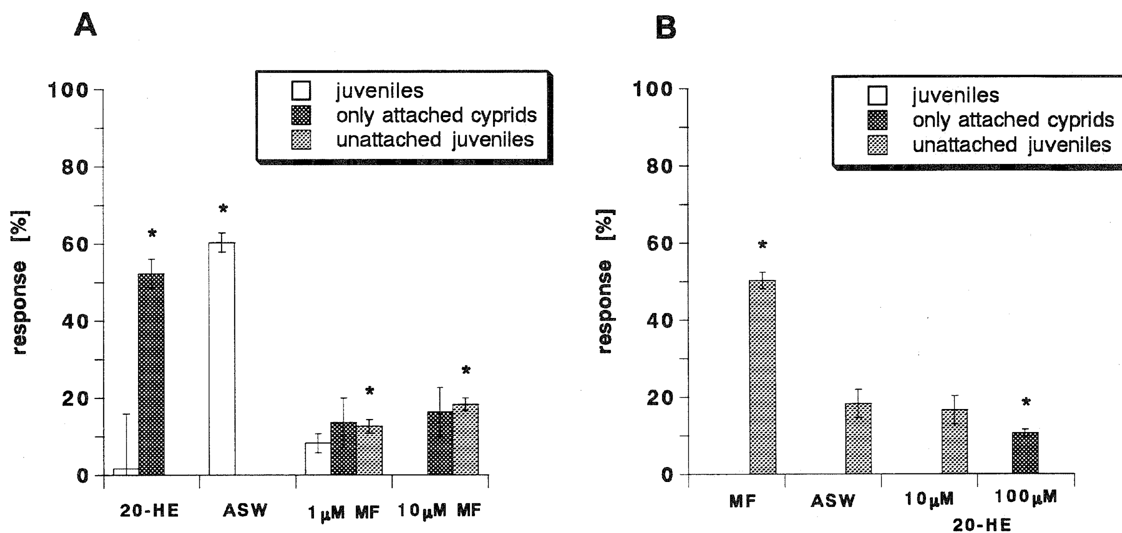

Fig. 3. Effect of a combination of 20-HE and MF. Data presented are means ± S.D. (**A**) 100 μM 20-HE pretreatment, (**B**) 10 μM MF pretreatment. ∗: significant at $p<0.05$.

niles. Most of them just attached to substrata and stopped metamorphosing; they were alive during our observation. Therefore, 20-HE induces attachment and metamorphosis at the lower concentrations, and inhibits metamorphosis at the high concentrations.

In order to examine the roles of 20-HE in larval metamorphosis, effects of a combination of MF and 20-HE were examined. After pretreatment with 100 μM 20-HE for 1 hr, cyprids were transferred into the wells, containing 100 μM 20-HE, ASW alone, 1 μM MF, and 10 μM MF respectively. Even after 20-HE pretreatment, MF induced metamorphosis (Fig. 3A). Statistical significance was as follows (ANOVA): juveniles; $F=153.0602$, $p<0.0001$, nonmetamorphosed, attached cyprids; $F=23.1932$, $p<0.0001$, unattached juveniles; $F=37.5754$, $p<0.0001$. Similarly, after pretreatment with 10 μM MF for 1 hr, cyprids were transferred into the wells, containing 10 μM MF, ASW alone, 10 μM 20-HE, and 100 μM 20-HE, respectively. Interestingly, 10 μM 20-HE did not cancel the effects of MF (Fig. 3B). Statistical significance was as follows: nonmetamorphosed, attached cyprids; $F=40.6349$, $p<0.0001$, unattached juveniles; $F=59.9914$, $p<0.0001$.

Table 1. Effects of 20-HE in cyprid attachment and metamorphosis when microinjected

		juvenile	nonmetamorphosed attached cyprid
20-HE	(2 μmol, n=12)	5	0
	(20 μmol, n=12)	1	4
	(200 μmol, n=12)	0	7
ASW	(n=9)	3	0

(μmol, n), (approximate internal agent concentration, sample size)

In order to check whether 20-HE could be entered in cyprids, we microinjected 20-HE into the inside of cyprids body. As shown in Table 1, a similar trend was obtained with microinjection experiments. When more than 20 μM of 20-HE were injected, metamorphosis was inhibited; most cyprids attached to the substrate, but did not metamorphose into juveniles.

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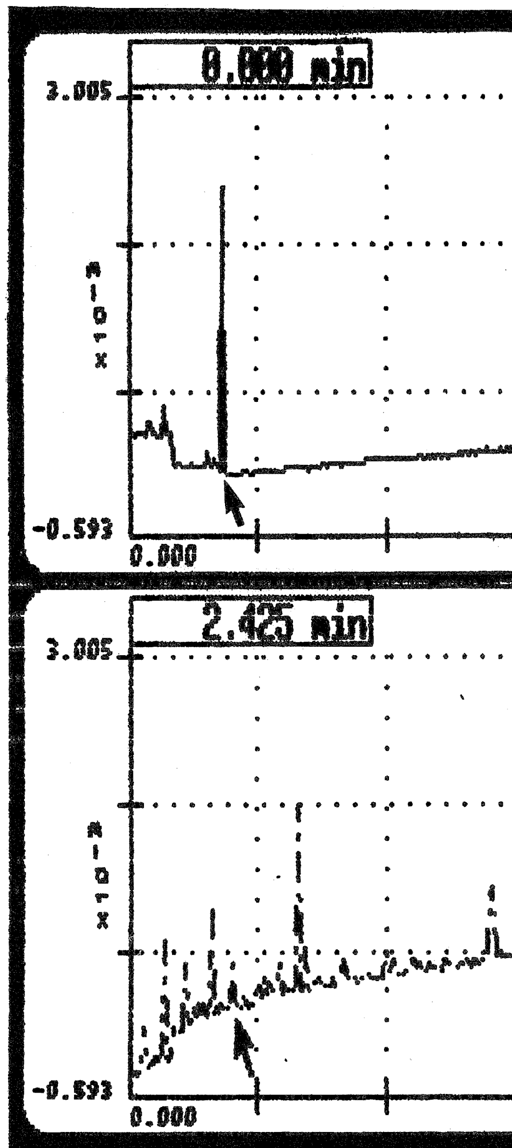

Fig. 4. High performance capillary electrophoresis profile of the fraction prepared from cyprid extract of *Balanus amphitrite*. Arrows indicate 20-HE peak. Upper, standard 20-HE; lower, extract from cyprids.

Extracts from cyprids were analyzed by HPCE using a P/ACE 2100 system. 20-HE was detected in cyprids at a concentrations of 2.2 pg/cyprid (Fig. 4).

DISCUSSION

Signal transduction systems involved in larval attachment and metamorphosis of sessile marine invertebrates are believed to proceed as follows: having located an appropriate settlement site, tactile stimuli signal larvae to stop swimming and to settle. There then follow internal signals which result in irreversible changes in overall body shape. It is widely perceived that chemoreception systems are associated with attachment, while signal transduction systems are employed during metamorphosis (Pawlik, 1992).

In barnacles, competent cyprid larvae initially swim then crawl in search of appropriate attachment substrata, where they eventually attach and metamorphose (Crisp, 1984; Walker *et al.*,1987). Larval attachment and metamorphosis are influenced by a range of environmental and artifical factors: attachment surface color (Yule and Walker,1984), water movement (Crisp,1955; Rittschof *et al.*,1984), conspecific adult-derived proteins (Larman *et al.*,1982), soluble pheromones (Rittschof, 1985; Kitamura, 1996), synthetic peptide analogs of barnacle settlement (attachment) pheromone (Tegtmeyer and Rittschof, 1986), footprints by cyprid antennular secretion (Walker and Yule, 1984; Yule and Walker, 1985; Clare *et al.*, 1994) and bacterial films (Maki *et al.*, 1988, 1990). Furthermore, Rittschof (1993) reviewed body odors and neutral-basic peptide mimics: responses by marine organisms. In addition to these exogenous factors, the involvement of signal transduction systems has also been reported. Rittschof *et al.* (1986) reported cAMP and dopamine involvement in settlement and Clare *et al.* (1995) reported cAMP involvement in attachment of *B. amphitrite.* In our previous studies, we reported that PKC signal transduction system is involved in metamorphosis (Yamamoto *et al.*,1995), and serotonergic neurons are associated with the attachment of *B. amphitrite* (Yamamoto *et al.*,1996).

Relatively little information of hormone-like substances in barnacles is available: Gomez *et al.* (1973) demonstrated that juvenile hormone mimics induced premature metamorphosis in the acorn barnacle *B. galeatus*. Tighe-Ford (1977) also reported the effects of juvenile hormone analogs on metamorphosis in *Elminus modestus* Darwin, while the relationship between the cyprid molt cycle and 20-HE in *B. amphitrite* was reported by Freeman and Costlow (1983). Mortlock *et al.* (1984) reported the effects of farnesol on the nauplius and cyprids of *E. modestus* Darwin, and recently Clare *et al.* (1992) reported the effects of the nonsteroidal ecdysone mimic RH 5849 on *B. amphitrite*. Recently, we reported that a hormonal substance, methyl farnesoate induce larval metamorphosis via PKC activation (Yamamoto *et al.*, 1997a).

Previously, we demonstrated that PKC activation by phorbol esters induced larval metamorphosis without attachment (Yamamoto *et al.*, 1995) and methyl farnesoate (MF) induced larval metamorphosis via PKC activation (Yamamoto *et al.*, 1997a). In the lobster *Homarus americanus*, MF stimulates the secretion of 20-HE (Chang *et al.*, 1993), and similarly ecdysteroid secretion from crab Y-organs in the crab *Cancer magister*, (Tamone and Chang, 1993). MF is involved in molting process in the giant freshwater prawn *Macrobrachium rosenbergii* (Wilder *et al.*, 1995). In the barnacle *B. amphitrite*, Freeman and Costlow (1983) reported that 20-HE was involved in cyprid molt cycle. They showed that 20-HE did not promote early premolt apolysis at the concentrations of 2-20 μM, while the integumental activities associated with postattachment metamorphosis were stimulated by 20-HE in a dose-dependent manner. Furthermore, we demonstrated that effects of a combination of 20-HE and MF on

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larval attachmant and metamorphosis (Fig. 3). When cyprids were exposed to 100 μM 20-HE alone, most of them attached to substrata but did not metamorphose into juveniles. However, treatment with 1 μM MF or 10 μM MF after pretreatment with 100 μM 20-HE reduced percentages of nonmetamorphosed, attached cyprids. Although these cyprids did not metamorphose, premolt apolysis occured partially. MF induced metamorphosis without attachment to substrata. These results suggest that effect of 100 μM 20-HE remain in cyprids even after MF treatment (Fig. 3A). When cyprids were exposed to 10 μM MF alone, most of them did not attach, but metamorphosed into juveniles. However, treatment with 10 μM 20-HE or 100 μM 20-HE after pretreatment with 10 μM MF reduced ratios of unattached juveniles in a dose dependent manner (Fig. 3B). Previously, the relationship between MF and 20-HE was reported by Chang *et al.* (1993), which demonstrated that MF stimulates the secretion of 20-HE in the lobster *H. americanus*. These results suggest that in *B. amphitrite* cyprids, when MF stimulates 20-HE secretion strongly, high concentrations of 20-HE suppresses metamorphosis mainly in molting, while MF stimulates 20-HE secretion slightly, low concentrations of 20-HE promotes attachment and metamorphosis. In addition to the results mentioned above, results of microinjection of cyprid larva showed dose-dependent effects similar to that with the routine plate assays (Table 1). Moreover, in order to confirm the roles of 20-HE in cyprids, we checked for the presence of this hormone in cyprids by HPCE. 20-HE was detected in extract from cyprids (Fig. 4).

In conclusion, our experiments demonstrate that 20-HE promoted attachment and metamorphosis at the concentrations of 0.001-0.1 μM on day 1. While 20-HE produced nonmetamorphosed, attached cyprids at the concentrations of 10-100 μM. These results suggest that 20-HE concentration in cyprids is associated with regulation of metamorphosis including molting in this species in the natural environment. Furthermore, we take our previous results into consideration (Yamamoto *et al.*, 1997a), we may conclude that cyprid metamorphosis, especially molting process is regulated by the balance of MF and 20-HE in this species.

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