INTRODUCTION

In Crustacea, the neuroendocrine X-organ and sinus gland complex in the eyestalk has long been known to play an important role in the regulation of molting and female reproduction (see reviews1–4). The roles of the X-organ-sinus gland complex were originally demonstrated by eyestalk ablation experiments in the early works of Zeleny,5 Smith6 and Panouse.7 As eyestalk ablation caused the stimulation of molting and female reproduction, the existence of inhibiting factors in the eyestalk was postulated. In recent studies, these inhibiting factors were purified as neuropeptide hormones: (e.g. molt-inhibiting hormone (MIH),8,9 vitellogenesis-inhibiting hormone (VIH),10 and mandibular organ-inhibiting hormone (MOIH)).11,12 The action of these hormones has been examined principally by in vitro studies. MIH inhibits secretion of molting hormone, ecdysteroid from the crustacean molting glands, the Y-organs.13,14 VIH is considered to inhibit vitellogenin synthesis at the vitellogenin synthetic site.15,16 MOIH acts on the mandibular organ, and inhibits secretion of methyl farnesoate (MF) which in turn is thought to stimulate vitellogenesis and Y-organ ecdysteroid secretion.17,18 Consequently, the hypothesis that MIH, VIH and MOIH play central roles in the regulation of molting and female reproduction has become well established (see reviews19,20).

However, in addition to the eyestalk neuropeptide hormones, the existence of other factors, such as vitellogenesis-stimulating hormone (VSH), vitellogenesis-stimulating ovarian hormone
(VSOH), and ovarian hormones controlling external female characteristics have been reported.\textsuperscript{21–27} Moreover, crustacean cardioactive peptide (CCAP) and negative feedback regulation of the Y-organ were found recently.\textsuperscript{28,29} In this way, regulatory mechanisms of molting and female reproduction are likely more complicated than discussion which is focused on eyestalk hormones only. Furthermore, in most crustacean species, females continue to molt after reaching sexual maturity, and many observations indicate the existence of mechanisms coordinating molting and female reproduction (see reviews\textsuperscript{13}). For example, females mate and spawn immediately after ecdisis, and females do not molt during the brooding of embryos. The above eyestalk hormones and other factors may also participate in this coordination of molting and female reproduction, but such mechanisms have not yet been clarified. Thus, re-examination of roles of the eyestalk hormones would be expected to provide useful and basic information on the regulatory mechanisms of molting and female reproduction.

The principal objective of this study was to examine the effects of bilateral eyestalk ablation on molt interval and hemolymph levels of ecdysteroid and vitellogenin as a first step in clarifying the roles of the eyestalk hormones in the regulation of molting and ovarian development in the giant freshwater prawn, \textit{Macrobrachium rosenbergii}. This species shows a distinct relationship between molting and female reproduction; in females, ovarian development and changes in vitellogenin levels occur synchronously with the molting process, and mating and oviposition occur immediately after ecdisis.\textsuperscript{30–32} For this reason, we also investigated the effects of ablation on the dynamics of molting and ovarian development in order to gain more insight into the roles of eyestalk hormones in coordinating these two processes.

\section*{MATERIALS AND METHODS}

\subsection*{Animals}

Mature male and female freshwater prawns \textit{M. rosenbergii} (28–41 mm in carapace length) were obtained from local aquaculture farmers in Osaka and Kumamoto Prefectures. Individual prawns were kept in divided chambers maintained at 28°C under a photoperiod regime of 15 L : 9 D, and were fed commercial pellets daily. The prawns were acclimated in the chambers for at least 2 weeks prior to use in experimentation. Under these conditions, females exhibited occurrence of the reproductive and non-reproductive molt cycles (common molt cycle) in a random fashion.\textsuperscript{30} The reproductive molt cycle was characterized by the occurrence of ovarian development culminating in oviposition and the appearance of ovigerous setae on the pleopods after ecdisis, whereas the non-reproductive molt cycle was not accompanied by ovarian development and oviposition.\textsuperscript{30–32}

\subsection*{Eyestalk ablation and hemolymph sampling}

In order to examine effects of hemolymph sampling on molt intervals and female molt types, experiments were conducted twice: experiment without hemolymph sampling and that with repeated sampling. The size of animals used was different between the experiments with and without hemolymph sampling due to different batches of animals (Tables 1 and 2). In both experiments, animals were divided into the destalked and control groups randomly. Thus, four groups were set up for both sexes as shown in Tables 1 and 2.

As ecdisis usually occurs at night,\textsuperscript{30} incidence of ecdisis was recorded the next morning (Day 1). Bilateral ablation of the eyestalks was carried out on Day 2 by simply holding the animal half-submerged in water, and snipping both eyestalks at the base using scissors. For the control group, no treatments were employed on Day 2. Molt interval until the next incidence of ecdisis and female molt type according to the above criteria were recorded for purposes of comparison between the control and destalked animals.

Hemolymph samples (50–80 \textmu L) were repeatedly taken from the pericardial cavity using a syringe with a 25G needle during the first molt cycle following eyestalk ablation on Days 2, 8, 12, 18, 24, and 30.

\begin{table}[h]
\centering
\caption{Duration of molt interval and carapace length in control and destalked males.}
\begin{tabular}{lccc}
\hline
\textbf{Group} & \textbf{No. individuals} & \textbf{Carapace length (mm)*} & \textbf{Molt interval (days)**} \\
\hline
Without sampling & & & \\
Control & 11 & 38.5 ± 2.2\textsuperscript{a} & 26.3 ± 1.5\textsuperscript{a} \\
Destalked & 13 & 38.5 ± 2.4\textsuperscript{a} & 15.0 ± 1.0\textsuperscript{b} \\
With sampling & & & \\
Control & 17 & 33.0 ± 2.7\textsuperscript{b} & 29.6 ± 2.3\textsuperscript{a} \\
Destalked & 15 & 34.1 ± 4.1\textsuperscript{b} & 15.1 ± 0.8\textsuperscript{b} \\
\hline
\end{tabular}
\begin{tablenotes}
\item \textsuperscript{*}Results are shown as the mean ± SD.
\item \textsuperscript{**}Results are shown as the mean ± SEM.
\item \textsuperscript{a,b}Means with the same superscript are not significantly different at the $P<0.05$ level.
\end{tablenotes}
\end{table}
Table 2  Duration of molt interval, carapace length and molt types in control and destalked females

<table>
<thead>
<tr>
<th>Group</th>
<th>No. individuals</th>
<th>Carapace length (mm)*</th>
<th>Molt interval (days)**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>RM</td>
<td>Non-RM</td>
</tr>
<tr>
<td>Without sampling</td>
<td>Control</td>
<td>51</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>Destalked</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>With sampling</td>
<td>Control</td>
<td>13</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Destalked</td>
<td>14</td>
<td>14</td>
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</tbody>
</table>

RM, reproductive molt cycle; non-RM, non-reproductive molt cycle.
*Results are shown as the mean ± SD.
**Results are shown as the mean ± SEM.
Means with the same superscript are not significantly different at the P<0.05 level.

20 and 27 for males and Days 2, 9, 16, 23 and 30 for females. Day 2 samples were taken just before eyestalk ablation on the same day. Some of the experimental prawns molted before the end of the sampling schedule. Hemolymph samples were not taken after ecdysis, as molted animals were under different physiological conditions (i.e. low hemolymph levels of ecdysteroids and vitellogenin). At conclusion of sampling, the non-molted animals were kept under observation until ecdysis in order to determine molt interval and female molt type. The samples were stored at −70°C until analysis of ecdysteroid levels for both sexes, and vitellogenin levels for females. Molt stages were determined at the time of sampling.

In addition, hemolymph samples were taken during the second molt cycle following eyestalk ablation from destalked males that were not used for hemolymph sampling in the first molt cycle. Sampling was carried out twice at molt stages C<sub>1</sub>-D<sub>0</sub> and D<sub>2</sub>-D<sub>3</sub>. The samples were stored at −70°C until ecdysteroid analysis.

Molt staging

Molt stages were determined based on setogenesis of the pleopod setae according to previously described criteria<sup>34,35</sup> with some modification. The distal part of the pleopods was clipped using scissors, and the degree of setogenesis in the setae was observed using a differential phase-contrast microscope. The molt cycle was divided into four stages (e.g. postmolt, intermolt, premolt and ecdysis). Each molt stage was further divided into substages: postmolt (A and B), intermolt (C<sub>0</sub> and C<sub>1</sub>), premolt (D<sub>0</sub>, D<sub>1</sub>, D<sub>2</sub> and D<sub>3</sub>) and ecdysis (E). In the present study, stage D<sub>3</sub> was further divided into D<sub>3</sub><sup>¢</sup> (completion of new barbule formation on new setae) and D<sub>3</sub><sup>¢¢</sup> (completion of new cuticle formation under the old cuticle).

Radioimmunoassay and high-performance liquid chromatography of ecdysteroids

A double antibody radioimmunoassay (RIA) method was employed after extraction with methanol in order to determine hemolymph ecdysteroid titer. The procedures and validation of this technique were as described previously.<sup>36</sup> The antiserum (provided by Prof. M. Nagata, the University of Tokyo) showed similar cross-reactivity toward 20-hydroxyecdysone (20E) and ecdysone (E). Control females exhibited both reproductive and non-reproductive molting, and as ecdysteroid levels between the two cycles were found not to be significantly different,<sup>33</sup> levels in control females were used for comparison with those of destalked females.

In order to characterize ecdysteroid species present in the hemolymph, samples for stage D<sub>3</sub><sup>¢</sup> were pooled and analyzed using a high-performance liquid chromatography (HPLC)-RIA system according to previously reported methods.<sup>36</sup> Briefly, hemolymph ecdysteroids were extracted with methanol and subjected to HPLC separation using a reversed-phase column (ODS-80TM; Tosoh Co. Ltd, Tokyo, Japan) and a methanol-acetonitrile–water solvent system. The ecdysteroid content in each fraction was quantified by RIA.

Vitellogenin enzyme-linked immunosorbent assay

Hemolymph vitellogenin levels were measured by a vitellogenin enzyme-linked immunosorbent assay (ELISA) according to procedures described previously.<sup>32,35</sup> Purified vitellin from <i>M. rosenbergii</i> was used as a standard, and antibody used in this assay was raised against purified vitellin from the freshwater prawn, <i>Macrobrachium nipponense</i>.<sup>34,37</sup>
Vitellogenin levels in reproductive females only were compared between the control and destalked groups, as ovaries developed only during the reproductive molt cycle.\textsuperscript{31,32}

\textbf{Statistics}

Data corresponding to molt interval and ecdysteroid and vitellogenin levels were analyzed statistically by the Mann–Whitney U-test for two-samples, the Wilcoxon’s signed ranks test for paired-samples and the Dunn test after Kruskal–Wallis test for multiple sample comparison. Statistical analysis of cumulative molting percentage was performed using the Fisher’s exact probability test. Results are expressed as the mean ± SEM.

\textbf{RESULTS}

\textbf{Molt interval in males}

In comparing molt interval between control and bilaterally destalked males (Table 1), destalked males showed significantly shortened molt interval ($P<0.01$). There was no significant difference in molt interval between hemolymph sampled and non-sampled animals ($P>0.05$), indicating that there were no significant effects of sampling on molt interval.

\textbf{Hemolymph ecdysteroid levels in males}

Destalked males showed a rapid increase in hemolymph ecdysteroid levels and a short molt cycle compared with control males (Fig. 1). In destalked males, hemolymph ecdysteroid levels increased from Day 2 to Day 12. Levels on Day 12 (34.1 ± 15.1 ng/mL) were significantly higher than those on Day 2 (0.46 ± 0.08 ng/mL, $P<0.01$). Subsequently, 13 of 15 males (87%) molted prior to Day 20 (Fig. 1), and only two males remained having not molted. Ecdysteroid levels in the two non-molted males on Day 20 were 7.9 and 120 ng/mL. In contrast, control males showed a longer molt cycle, and cumulative molting percentage (18%) on Day 20 of control males was significantly lower than that of destalked males ($P<0.01$). Hemolymph ecdysteroid levels in control males increased relatively slowly (i.e. levels were low on Days 2, 8 and 12 (0.34–0.67 ng/mL) and increased on Days 20 and 27 (5.6 ± 3.3 and 55.7 ± 24.1 ng/mL, respectively). However, there was no significant difference in level, probably due to high variance ($P>0.05$). Levels on Days 8 and 12 in destalked males were significantly higher than those in control males ($P<0.01$).

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig1}
\caption{Cumulative molting percentage (a) and fluctuations in hemolymph ecdysteroid levels in terms of days after ecdysis (b) in control and destalked male \textit{Macrobrachium rosenbergii}. The number of samples decreased as cumulative molting percentage increased, as ecdysteroid levels of animals after ecysis were not included in data calculations. Points and vertical bars indicate the mean ± SEM ($n=10–17$), and points of destalked males on Day 20 indicate individual data ($n=2$). Arrows indicate the timing of bilateral eyestalk ablation on Day 2. * Indicates significant differences between control and destalked animals ($P<0.05$).}
\end{figure}

Figure 2 indicates the relationship between ecdysteroid levels and molt stage. Destalked and control males showed similar fluctuations (i.e. levels were low during stages B to D\textsubscript{0}, increased gradually at stages D\textsubscript{1}–D\textsubscript{2}, reached a large peak at stage D\textsubscript{3}, and then declined sharply at stage D\textsubscript{3} just prior to ecysis). There was no significant difference in levels at each molt stage between control and destalked males ($P>0.05$).

Six of the 13 destalked males that were not hemolymph sampled completed the second molt cycle after eyestalk ablation, and interval of the second molt cycle was also significantly shortened (21.5 ± 1.7 days, $P<0.01$) in comparison with molt interval of control males. In the six destalked
males, the second molt interval was significantly longer than the first molt interval (14.2 ± 0.8 days, \( P < 0.05 \)). Hemolymph ecdysteroid levels during the second molt cycle were determined using four of the six males that completed the second molt cycle. Levels were 0.43 ± 0.12 ng/mL at stages C1-D0, and then became significantly high at stages D2-D3 (26 ± 19 ng/mL, \( P < 0.05 \)), indicating that this pattern of ecdysteroid fluctuation was similar to that of control males as shown in Fig. 2a.

Reproductive molt frequency and molt interval in females

In the control females, the percentages of animals undergoing a reproductive molt cycle were 69 and 51% with and without hemolymph sampling and, in contrast, all bilaterally destalked females exhibited a reproductive molt cycle even under continuous hemolymph sampling (Table 2). In destalked females, ovarian development proceeded during the molt cycle and culminated in oviposition just after ecdysis, and ovigerous setae appeared on the pleopods after ecdysis in a similar manner to that of control females. The duration of molt interval was significantly shortened in destalked females undergoing reproductive ecdysis compared to that of control females in the reproductive molt cycle (\( P < 0.01 \), Table 2). This result also indicates that the period of the ovarian development was shortened in destalked females, because oviposition (i.e. the culmination of the ovarian development) occurred following the reproductive ecdysis in these females as well. In addition, the extent to which molt interval was shortened was larger in males than in females. There was no significant difference in reproductive molt cycle frequency or molt interval between non-sampled females and females which were repeatedly sampled (\( P > 0.05 \)), indicating that the repeated sampling did not influence female molt type or molt interval significantly.

Hemolymph ecdysteroid levels in females

Destalked females showed a rapid increase in ecdysteroid levels as did destalked males (Fig. 3). In destalked females, ecdysteroid levels were low on Days 2 and 9 (0.87 and 1.04 ng/mL, respectively), and increased to significantly high levels on Day 16 (46.8 ± 19.5 ng/mL, \( P < 0.01 \)). Twelve of 14 destalked females (86%) molted prior to Day 23, and only two females remained having not molted. Ecdysteroid levels in the two non-molted females on Day 23 were 2.2 and 248 ng/mL. In contrast, control females showed a slow rise in ecdysteroid levels. Levels were low on Days 2 and 9 (0.94 and 0.47 ng/mL, respectively), increased gradually on Days 16 and 23 (4.3 and 4.6 ng/mL, respectively) and finally reached significantly high levels on Day 30 (61.7 ± 29.6 ng/mL, \( P < 0.05 \)). The cumulative molting percentage (15%) on Day 23 of control females was significantly lower than that of destalked females (\( P < 0.01 \)). Levels on Day 16 in destalked females were significantly higher than those in control females (\( P < 0.01 \)).

The relationship between ecdysteroid levels and molt stage in females is shown in Fig. 2b. There was no significant difference in levels between the control and destalked females (\( P > 0.05 \)). This is similar to that found in males.

Hemolymph vitellogenin levels in females

Figure 4a shows fluctuations in hemolymph vitellogenin levels following ecdisis. Only levels in
hemolymph vitellogenin levels increased from stages B-C to C1, remained at high titers during stages C1-D2, and declined in stages D2-D3. There was no significant difference in levels between both groups (P > 0.05).

High-performance liquid chromatography–radioimmunoassay analysis of ecdysteroid

Ecdysteroid species in hemolymph samples at stage D3 were analyzed by HPLC-RIA (Fig. 5). In all
four groups (e.g. control and destalked males and control and destalked females) two peaks were identified as 20E and E, respectively, by coelution with authentic standards. 20E was the predominant ecdysteroid in all groups. Two unknown peaks were detected prior to 20E and were termed high polarity products (HPP). In the control and destalked females, one minor peak was detected at a similar elution position to that of inokosterone. In destalked males, there were two minor peaks that showed similar retention times to those of ponasterone A (PoA) and 20-hydroxyecdysone-22-acetate (20E22Ac), respectively.

**DISCUSSION**

In this study, eyestalk ablation induced stimulation of ovarian development, shortening of molt interval and rapid increases in ecdysteroid levels in *M. rosenbergii*. These results can be considered as the effects of the absence of eyestalk hormones, MIH, VIH and MOIH due to ablation, as discussed in the following.

Eyestalk ablation during molt stages B-C₀ caused a 100% occurrence of the reproductive molt cycle accompanied by ovarian development and oviposition compared with 51–69% in control females. This indicates that it is undetermined during molt stages B-C₀ whether females will commence ovarian development (reproductive molt cycle) or not (non-reproductive molt cycle) and that eyestalk ablation induces them to undergo ovarian maturation. Furthermore, yolk accumulation is initiated at stage C₁ in intact mature females, suggesting that differentiation towards reproductive or non-reproductive molting can occur prior to the C₁ stage. Consequently, the B-C₀ stages may be a critical period for the determination of reproductive molting.

In addition, the duration of ovarian development was shorter in destalked females than in control females. This result indicates that eyestalk ablation accelerates the process of ovarian development. However, vitellogenin levels were not significantly different between destalked and control reproductive females. This may be due to the sampling schedule employed in this study. As we did not take hemolymph samples for a week following eyestalk ablation in order to minimize stress due to the loss of hemolymph, it is possible that earlier increases in vitellogenin levels in destalked females during Days 3–8 were not detected.

As ablation is expected to remove the source of VIH and MOIH, decreases in hemolymph levels of VIH and/or MOIH at stages B-C₀ is likely to be a basis for the 100% occurrence of the reproductive molt cycle and the acceleration of ovarian development. Roles of VIH and MOIH on regulation of ovarian development have been partially understood. Vitellogenin is synthesized by the hepatopancreas, transported via hemolymph into the ovary, and accumulated in the oocytes as vitellin. VIH is considered to inhibit the vitellogenin synthesis in the hepatopancreas and/or the vitellogenin uptake into the oocytes (see for reviews). The absence of inhibitory regulation by VIH may therefore accelerate ovarian development. Furthermore, decreases in MOIH levels may accelerate ovarian development through a rise in levels of MF which is known to have stimulatory effects on ovarian development. This hypothesis is supported by a previous report that the eyestalk ablation induced a rise in hemolymph MF.
levels and stimulation of ovarian development in female \textit{Libinia emarginata}.\textsuperscript{41} However, functioning of MF in crustacean reproduction does not seem to be universal.\textsuperscript{42} In \textit{M. rosenbergii}, MF levels were not significantly different between reproductive and non-reproductive females\textsuperscript{43} and, thus, the roles of MOIH and MF in the regulation of female reproduction are not clear in this species.

In addition to VIH and MF, involvement of ecdysteroids in the regulation of female reproduction has been indicated by several reports (see for reviews\textsuperscript{3,4}). As hemolymph ecdysteroid levels are controlled principally by MIH,\textsuperscript{2} eyestalk ablation possibly affected through removal of MIH source. However, in \textit{M. rosenbergii}, ecdysteroids do not seem to be involved in regulating the mechanism of female reproduction, as hemolymph ecdysteroid levels were not different between the reproductive molt cycle concomitant with ovarian development and the non-reproductive molt cycle.\textsuperscript{33} Thus, MIH probably does not play an important role in the regulation of female reproduction in this species.

Eyestalk ablation also caused shortening of molt interval and increase in hemolymph ecdysteroid levels in \textit{M. rosenbergii}. These results are consistent with those of previous reports (see reviews\textsuperscript{1,2}). These results provide the following scheme as described previously.\textsuperscript{20} In the premolt stages, decreases in hemolymph MIH levels stimulate ecdysteroid secretion from the Y-organ and, as a result, hemolymph ecdysteroid levels begin to increase. At stage D\textsubscript{3}, an increase in MIH levels reduces secretory activity in the Y-organ, and then ecdysteroid levels decline sharply. MF is known to stimulate ecdysteroid secretion from the Y-organ in the crab \textit{Cancer magister},\textsuperscript{18} and MF levels become high at the premolt stages in \textit{M. rosenbergii},\textsuperscript{43} suggesting that MF also participates in the regulation of molting. Thus, the absence of MOIH in ablated animals may be involved in affecting the observed increases in ecdysteroid levels.\textsuperscript{44}

However, even after eyestalk ablation, six of 13 males repeated the molt cycle twice. Six of 14 destalked females also repeated the molt cycle twice (data not shown). The destalked males showed a decrease in hemolymph ecdysteroid levels at molt stage D\textsubscript{7}” during the first molt cycle and an increase in levels at the premolt stages during the second molt cycle. This normal fluctuation was undergone in the absence of the regulation of MIH or MOIH. Other authors have reported similar results. For example, bilaterally destalked animals undergo more than two molt cycles in the American lobster, \textit{Homarus americanus} and the banded coral shrimp, \textit{Stenopus hispidus}.\textsuperscript{35,46} Destalked lobsters showed a typical pattern of hemolymph ecdysteroid level fluctuation.\textsuperscript{45} If ecdysteroid fluctuations and molt cycle are regulated only by MIH and MOIH, it cannot be explained how they are controlled in the destalked animals.

Furthermore, the correlation of female reproduction to molting cycle persisted even after eyestalk ablation. In destalked females, oviposition occurred and ovigerous setae appeared on the pleopods after ecdisis. The relationship between vitellogenin levels and molt stage was also similar between destalked and control reproductive females. The extent of the shortening of molt interval by the eyestalk ablation was smaller in females than in males, indicating that molt intervals could be adjusted to accommodate ovarian development in the molt cycle even in destalked females. If the eyestalk hormones regulate the correlation of female reproduction and molting, these observations in the destalked females could not be explained.

In order to explain the continuous molt cycle and the correlation of female reproduction and molting in the destalked animals, it is probably necessary to postulate the involvement of undetermined endocrine factors except for the eyestalk hormones. As regulatory factors on molting and female reproduction, several factors have been reported in addition to the eyestalk hormones. Negative feedback control of hemolymph ecdysteroid, where circulating ecdysteroid inhibits Y-organ activity, was recently found in the crayfish \textit{Orconectes limosus}.\textsuperscript{29} Negative feedback control may act to maintain low hemolymph ecdysteroid levels in the intermolt stages and to drop levels suddenly in the late premolt stages. Recently, Philippen \textit{et al.}\textsuperscript{28} found that CCAP is dramatically released into the hemolymph at the time of ecdisis in the crab \textit{Carcinus maenas} and in \textit{O. limosus}, suggesting involvement of CCAP in the molting regulation. In the regulation of ovarian development, the existence of stimulating factors, such as VSH in the thoracic ganglion and brain and VSOH have been reported, although they have not yet been isolated.\textsuperscript{21–23} VSH and/or VSOH may participate in the regulatory mechanism of the correlation of female reproduction and molt cycle. Furthermore, in the regulation of external female characteristics, ovarian hormones have been reported.\textsuperscript{24–27} The appearance of the ovigerous setae in the destalked females can be explained by effects of the ovarian hormones. These endocrine factors may play important roles in the regulatory mechanisms of molting and female reproduction. However, we did not determine roles of these factors in the
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destalked animals. Details of the mechanisms are required to be elucidated.

No destalked animals finish the third molt cycle after the ablation in this study. Almost half of the destalked animals died during the second molt cycle. Probably, eyestalk ablation disturbed most of the metabolic processes, because eyestalks include several important hormones such as crustacean hyperglycemic hormone (CHH), red pigment

concentrating hormone (RPCH), and pigment-dispersing hormone (PDH) in addition to MIH, VIH, and MOIH (see a review39). The second molt cycle of the destalked males was still shortened but was significantly longer than the first molt cycle in the destalked males. This is perhaps because long disturbance of the metabolic process resulted in the prolongation of the second molt cycle.

In HPLC-RIA analysis of hemolymph ecysteroids, 20E, the active ecysteroid species in Crustacea, was predominant and small amounts of E and HPP were detected in both control and destalked M. rosenbergii. In destalked males only, minor peaks coeluted with 20E22Ac and PoA standards, but were not further characterized in the present study. However, a significant role in molting could not be attributed to 20E22Ac and PoA due to their trace levels. These results indicate that ecysteroid metabolism is not changed significantly in destalked animals. Hemolymph HPP was not further identified in this study. However, the presence of 20-hydroxyecdysonic acid, ecdysonic acid, conjugated forms of ecysteroids, and 20,26-dihydroxyecdysonone was reported in the hemolymph, ovaries and eggs of M. rosenbergii.31,47–50

In the present study, we examined the effects of bilateral eyestalk ablation on molting and female reproduction in M. rosenbergii. The shortening of molt interval and the stimulation of ovarian development can be explained by the absence of the eyestalk hormones such as MIH, VIH, and MOIH by destalking. We also found that destalked males repeatedly underwent molting cycles and that the relationship between vitellogenin levels and molt stage persisted even after eyestalk ablation in females. These results suggest the existence of regulating factors in addition to the eyestalk hormones. Further studies are necessary in order to clarify the mechanisms of regulation of molting and female reproduction.

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