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# *In vivo* Effects of a Recombinant Molt-Inhibiting Hormone on Molt Interval and Hemolymph Ecdysteroid Level in the Kuruma Prawn, *Marsupenaeus japonicus*

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**ABSTRACT**—In order to determine the function of molt-inhibiting hormone (MIH) *in vivo*, we examined the effects of injecting of a recombinant MIH on the molt interval and hemolymph ecdysteroid level in the kuruma prawn, *Marsupenaeus japonicus*. The injection of recombinant MIH significantly prolonged the molt interval (9.0±0.4 days in the control group, 9.5±0.5 days in the 2500 ng/g-body weight/injection-group, mean±SD), and significantly decreased the hemolymph ecdysteroid level (ratio of levels between after and before injection: 1.94±1.09 in the control and 1.28±0.39 in the 3000 ng/g-body weight/injection-group, mean±SD). These results conclusively show the inhibitory effects of MIH on molting *in vivo*.

**Key words:** Crustacea, ecdysteroid, molt-inhibiting hormone, molting

## INTRODUCTION

Crustacean species grow by shedding the exoskeleton, i.e., molting. The process of molting is under hormonal control. The most widely accepted hypothesis for molting control is as follows (Skinner, 1985): Molting is triggered by ecdysteroids which are secreted by the Y-organ. The secretion of ecdysteroids is negatively regulated by the molt-inhibiting hormone (MIH), a neuropeptide, which is synthesized and secreted by the X-organ/sinus gland complex, a neurohemal organ. In this hypothesis, MIH plays a central role in molting control.

In several crustacean species, MIHs have been purified, and their amino acid sequences have been determined (reviewed by Keller, 1992; De Kleijn and Van Herp, 1995). The MIHs consist of 75–77 amino acid residues and contain six cysteine residues with three intrachain disulfide bridges. They belong to the crustacean hyperglycemic hormone (CHH) family, a neuropeptide group. The direct function of MIH on the Y-organ has been established by *in vitro* studies using cultured Y-organs (Mattson, 1986; Sedlmeier and Seinsche, 1998).

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For determining the function of MIH *in vivo*, most studies have used crude extracts of sinus glands (Bruce and Chang, 1984; Snyder and Chang, 1986), and there are few studies using purified MIH (Nakatsuji and Sonobe, 2004). This is mainly because it is difficult to purify sufficient amounts of MIH from the sinus gland extracts for *in vivo* administration. Furthormore, it is ambiguous if the effects of crude sinus gland extracts indicate the functions of MIH, because crude extracts contain several neuropeptides. Recently, a recombinant MIH (rMIH, Ohira *et al*., 1999) and a chemically synthesized MIH (Kawakami *et al*., 2000) have became available. These MIHs can be provided in sufficient amounts for *in vivo* studies. Using the synthetic MIH, its inhibitory effects on molting were determined *in vivo* in the American crayfish, *Procambarus clarkii* (Nakatsuji and Sonobe, 2004). This is the only study that has demonstrated *in vivo* effects of MIH clearly. For further understanding of the physiological roles of MIH, information on the *in vivo* effects of MIH should be accumulated.

The MIH of the kuruma prawn, *Marsupenaeus japonicus* was isolated as a peptide with 77 amino acid residues (Yang *et al*., 1996), and its rMIH was produced (Ohira *et al*., 1999). The effects of the purified MIH and rMIH of *M. japonicus* were determined *in vitro* using the crayfish Y-organ incubation assay but were not examined *in vivo* (Yang *et al*., 1996; Ohira *et al*., 1999). Thus, the aim of the present study is to determine the effects of *M. japonicus* MIH *in vivo*. We

injected the rMIH into prawns and determined the effects on molt interval and hemolymph ecdysteroid level.

## MATERIALS AND METHODS

### Animals

Juvenile *M. japonicus* were obtained from the Mie Prefectural Fish-Farming Center, Mie, Japan for Experiment 1 and from the Kamiura National Center for Stock Enhancement, Ooita, Japan for Experiment 2. Juvenile prawns were kept in sea water and were fed commercial pellets daily.

### Experiment 1: effects on molt intervals

Juvenile prawns (body weight (BW), 1.86–2.84 g) were kept individually in compartments (20×15×18 $cm^3$) with recirculating sea water (flow rate, 250 ml/min) at 24.9–25.1°C. Prawns were randomly divided into three groups of different doses: control (0 ng/g-BW), 250 ng/g-BW, and 2500 ng/g-BW. Recombinant MIH of *M. japonicus* (Ohira *et al*., 1999) was dissolved in shrimp saline (400 mM NaCl, 23 mM $MgSO_4$, 20 mM $MgCl_2$, 13 mM KCl, 8.6 mM $CaCl_2$, 3.6 mM $NaHCO_3$, and 0.37 mM $NaHPO_4$) to give concentrations of 50 and 500 ng/μl. The rMIH solutions were injected into the pericardial cavity (5 μl/g-BW) at 11:00–12:00 once a day on 2nd, 3rd, 4th, 5th, 6th, and 7th days after ecdysis. Prior to injection to each prawn, the rMIH solutions were mixed well, and it was confirmed that no visible precipitate was formed. The control prawns received an equal volume of saline. After six successive injections, the next ecdysis was checked every morning to determine molt intervals, because ecdysis usually occurs at night. Thus, the molt interval is a discontinuous variable, and the frequency distribution is shown in Results.

As a preliminary study, time-course change of the molt stage during the molt cycle was examined. Molt stages were determined by observing the setal development of the pleopods under a light microscope according to previously reported criteria (Okumura *et al*., 1992). The molt interval was 9.1±0.5 days (mean±SD), and prawns were at the premolt stages A–B on 1st day after ecdysis, at the intermolt stage C on 2nd and 3rd days, at the premolt stage $D_0$ on 4th day, at the premolt stage $D_1$ on 5th–7th days, and at the premolt stage $D_2$–$D_3$ on 8th day. Ecdysis occurred during the night of 9th day. Thus, the above injections were conducted in prawns at molt stages C–$D_1$, when hemolymph ecdysteroid levels start to increase (Okumura *et al*., 1989).

### Experiment 2: effects on hemolymph ecdysteroid levels

Juvenile prawns (BW, 1.76–3.32 g) were kept at 22.5–23.5°C. Because hemolymph ecdysteroid levels start to increase at the early premolt stage (Okumura *et al*., 1989), prawns at the premolt stage $D_1$ were selected and were randomly divided into three groups of different doses: control (0 ng/g-BW), 300 ng/g-BW, and 3000 ng/g-BW. Solution of rMIH was prepared as described above. A hemolymph sample (15–20 μl) was taken from each prawn using a syringe and needle from the pericardial cavity three hours before injection. The rMIH solution was injected into the pericardial cavity (6 μl/g-BW). The control prawns received an equal volume of saline. Twenty-one hours after the injection, the second hemolymph sample was taken. To avoid possible fluctuations in ecdysteroid levels over a 24 h period (Snyder and Chang, 1991), both hemolymph samples were taken at the same time of day. The hemolymph samples were stored at –80°C until analysis.

Ecdysteroids in the hemolymph samples were extracted with methanol according to a previously reported procedure (Okumura *et al*., 1989). The extracted ecdysteroids were measured by a competitive enzyme-immunoassay (EIA) according to a previously reported procedure (Porcheron *et al*., 1989). Anti-20-hydroxyecdysone antiserum and tracer (20-hydroxyecdysone-6-carboxymethyloxime-acetylcholinesterase conjugate) were purchased from Cayman Chemical (Ann Arbor, MI, USA). For the assay standard, 20-hydroxyecdysone was used, because the major hemolymph ecdysteroid in *M. japonicus* is 20-hydroxyecdysone (Okumura *et al*., 1989).

### Statistical analysis

Data of molt intervals were analyzed with the chi-square test. Data of ecdysteroid levels were analyzed with nonparametric statistical methods, because they exhibited a non-normal distribution. For paired comparisons, the Wilcoxon's signed ranks test was used. For multiple comparisons, overall analysis was done by the Kruskal-Wallis test, followed by the Steel-Dwass test when an overall significance was detected.

## RESULTS AND DISCUSSION

Fig. 1 shows the effects of rMIH injection on molt intervals. The molt interval was 9.0±0.4 days (mean±SD) in the control group, 9.2±0.6 days in the 250 ng/g-BW group, and 9.5±0.5 days in the 2500 ng/g-BW group. The molt interval significantly differed between the control and the 2500 ng/g-BW group ($P<0.05$).

Fig. 2 shows the effects of rMIH injection on hemolymph ecdysteroid levels. In all three groups, levels increased between before and after injection (in 24 h) significantly ($P<0.05$, Fig. 2A). Ratio of ecdysteroid levels between after and before injection was significantly lower in the 3000 ng/g-BW group (1.28±0.39, mean±SD) than in the control group (1.94±1.09) ($P<0.05$, Fig. 2B).

In the present study, we found inhibitory effects of rMIH on molt intervals and hemolymph ecdysteroid levels *in vivo* in *M. japonicus*. This is the first report that shows that *M. japonicus* MIH has inhibitory effects on molting in this species, because the *in vitro* effects of *M. japonicus* MIH have been examined using a hetero-species Y-organ incubation assay (Yang *et al*., 1996; Ohira *et al*., 1999). The MIH injec-

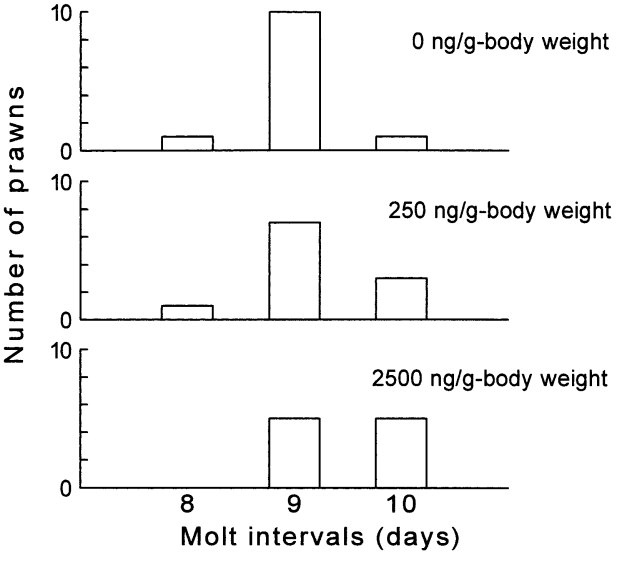


**Fig. 1.** Molt intervals of the control and rMIH-injected juvenile *M. japonicus*.

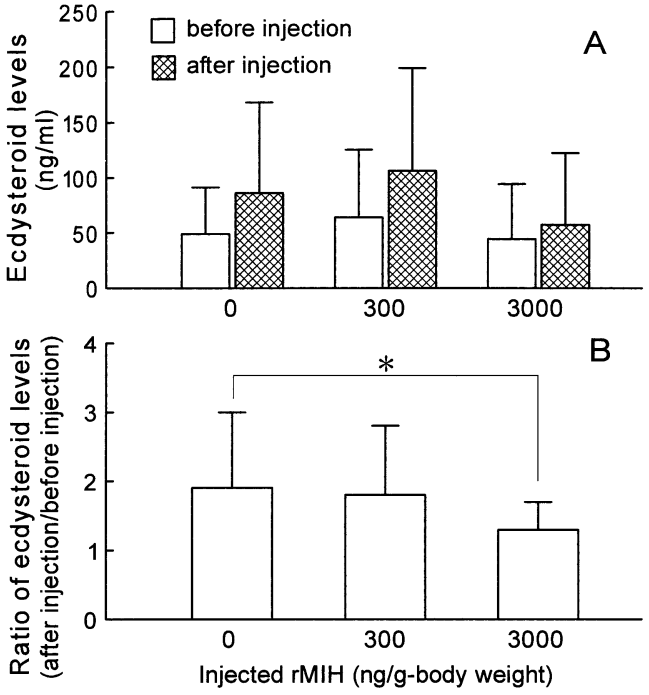


**Fig. 2.** Hemolymph ecdysteroid levels before and after injection of rMIH (A) and ratio of ecdysteroid levels between before and after injection (B) in juvenile *M. japonicus* at the premolt stage $D_1$. Data are expressed as mean±SD. The number of animals in each group was 17–18. Asterisk indicates a significant difference ($P$<0.05).

tion probably resulted in a decrease in hemolymph ecdysteroid levels first, and the delay of the ecdysteroid peak could cause the prolongation of the molt interval.

The timing of MIH injection in the molt cycle may be important for determining the effects of MIH, because hemolymph MIH levels and responsiveness of the Y-organ to MIH is known to change during the molt cycle. In Experiment 1, the injections of rMIH were carried out on prawns at molt stages C–$D_1$. Hemolymph MIH levels are high at intermolt stage C and decrease during the early premolt stages $D_0$–$D_1$ (Nakatsuji and Sonobe, 2003). The injected rMIH could compensate for the decrease in endogenous MIH levels and cause a delay in Y-organ activation at early premolt stage. Similarly, in Experiment 2, the rMIH injection was carried out on prawns at premolt stage $D_1$, when hemolymph MIH levels start to decrease. The injected rMIH could inhibit Y-organ activation by compensating for the decrease in endogenous MIH levels. The responsiveness of the Y-organ to MIH is high during postmolt, intermolt and early premolt stages, and becomes low during middle and late premolt stages (Sefiani *et al*., 1996; Chung and Webster, 2003; Nakatsuji and Sonobe, 2004). The rMIH injection was carried out during the period of high responsiveness of Y-organ.

The effects of the MIH injection were significant but small (5.6% increase in molt interval and 34% decrease in ecdysteroid level ratio). Nakatsuji and Sonobe (2004) reported a 27% increase in molt interval by MIH injection in *P. clarkii*. Injection of MIH was carried out on non-eyestalk-ablated prawns in the present study but on eyestalk-ablated crayfish in the previous study (Nakatsuji and Sonobe, 2004). The eyestalk-ablated crayfish do not have endogenous MIH, and may show a higher responsiveness to injected MIH than the non-ablated ones. Although injection to eyestalk-ablated animals is suitable to determine the effects of MIH, we did not use eyestalk-ablated prawns due to the high mortality previously observed to be caused by repeated injections after eyestalk-ablation in *M. japonicus*. We injected six times in six days, while Nakatsuji and Sonobe (2004) injected 20 times at 30 min intervals. Exogenous MIH is rapidly degraded *in vivo* (half-life of less than 10 min; Chung and Webster, 2003; Nakatsuji and Sonobe, 2004). The protocol of 20-times injection at 30 min intervals could be more effective at inducing the prolongation of the molt interval.

The rapid degradation of exogenous MIH (Chung and Webster, 2003; Nakatsuji and Sonobe, 2004) may also be the main reason why large amount of MIH (2500 or 3000 ng/g-BW/injection in the present study; 20-times injection of 250 pmol/animal, approximate total injected amount = 2200 ng/g-BW in Nakatsuji and Sonobe, 2003) is necessary to inhibit molting *in vivo*. The amounts of injected MIH (2200-3000 ng/g-BW/injection) are much higher than the levels in the hemolymph of *P. clarkii* (1.25–6.53 fmol-MIH/ml, (11–56 pg/ml), Nakatsuji and Sonobe, 2003). Some techniques for continuous administration of peptides, such as an osmotic pump, may be effective in enhancing the effects of exogenous MIH.

The present study demonstrates the inhibitory role of MIH *in vivo*. Recent studies on hemolymph MIH levels and Y-organ responsiveness to MIH suggest that the molt cycle is regulated by changes in the responsiveness of the Y-organ in addition to changes in hemolymph MIH levels (Chung and Webster, 2003; Nakatsuji and Sonobe, 2004). Furthermore, eyestalk-ablated (absence of MIH) prawns have been shown to repeat the molt cycle and showed a typical pattern of hemolymph ecdysteroid levels (Okumura and Aida, 2001). The classic hypothesis that focuses on synthesis and release of MIH should be re-examined, and more detailed studies on the Y-organ responsiveness are necessary to clarify the functioning of MIH in crustaceans.

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## REFERENCES

Bruce MJ, Chang ES (1984) Demonstration of a molt-inhibiting hormone from the sinus gland of the lobster, *Homarus americanus*. Comp Biochem Physiol 79A: 421–424

Chung JS, Webster SG (2003) Moult cycle-related changes in biological activity of moult-inhibiting hormone (MIH) and crusta-

cean hyperglycaemic hormone (CHH) in the crab, *Carcinus maenas*. Eur J Biochem 270: 3280–3288

De Kleijn DPV, Van Herp F (1995) Molecular biology of neurohormone precursors in the eyestalk of Crustacea. Comp Biochem Physiol 112B: 573–579

Kawakami T, Toda C, Akaji K, Nishimura T, Nakatsuji T, Ueno K, Sonobe M, Sonobe H, Aimoto S (2000) Synthesis of a molt-inhibiting hormone of the American crayfish, *Procambarus clarkii*, and determination of the location of its disulfide linkages. J Biochem 128: 455–461

Keller R (1992) Crustacean neuropeptides: structures, functions and comparative aspects. Experientia 48: 439-448

Mattson MP (1986) New insights into neuroendocrine regulation of the crustacean molt cycle. Zool Sci 3: 733–744

Nakatsuji T, Sonobe H (2003) Measurement of molt-inhibiting hormone titer in hemolymph of the American crayfish, *Procambarus clarkii*, by time-resolved fluoroimmunoassay. Zool Sci 20: 999–1001

Nakatsuji T, Sonobe H (2004) Regulation of ecdysteroid secretion from the Y-organ by molt-inhibiting hormone in the American crayfish, *Procambarus clarkii*. Gen Comp Endocrinol 135: 358–364

Ohira T, Nishimura T, Sonobe H, Okuno A, Watanabe T, Nagasawa H, Kawazoe I, Aida K (1999) Expression of a recombinant molt-inhibiting hormone of the kuruma prawn *Penaeus japonicus* in *Escherichia coli*. Biosci Biotechnol Biochem 63: 1576–1581

Okumura T, Aida K (2001) Effects of bilateral eyestalk ablation on molting and ovarian development in the giant freshwater prawn, *Macrobrachium rosenbergii*. Fisheries Sci 67: 1125–1135

Okumura T, Han CH, Suzuki Y, Aida K, Hanyu I (1992) Changes in hemolymph vitellogenin and ecdysteroid levels during the reproductive and non-reproductive molt cycles in the freshwater prawn *Macrobrachium nipponense*. Zool Sci 9: 37–45

Okumura T, Nakamura K, Aida K, Hanyu I (1989) Hemolymph ecdysteroid levels during the molt cycle in the kuruma prawn *Penaeus japonicus*. Nippon Suisan Gakkaishi 55: 2091–2098

Porcheron P, Moriniere M, Grassi J, Pradelles P (1989) Development of an enzyme immunoassay for ecdysteroids using acetylcholinesterase as label. Insect Biochem 19: 117–122

Sedlmeier D, Seinsche A (1998) Ecdysteroid synthesis in the crustacean Y-organ: role of cyclic nucleotides and $Ca^{2+}$. In "Recent Advances in Arthropod Endocrinology" Ed by GM Coast, SG Webster, Cambridge Univ Press, Cambridge, pp 125–137

Sefiani M, Le Caer JP, Soyez D (1996) Characterization of hyperglycemic and molt-inhibiting activity from sinus glands of the penaeid shrimp *Penaeus vannamei*. Gen Comp Endocrinol 103: 41–53

Skinner DM (1985) Molting and regeneration. In "The Biology of Crustacea Vol 9" Ed by DE Bliss, LH Mantel, Academic Press, New York, pp 43–146

Snyder MJ, Chang ES (1986) Effects of sinus gland extracts on larval molting and ecdysteroid titers of the American lobster, *Homarus americanus*. Biol Bull 170: 244–254

Snyder MJ, Chang ES (1991) Ecdysteroids in relation to the molt cycle of the American lobster, *Homarus americanus*. I. Hemolymph titers and metabolites. Gen Comp Endocrinol 81: 133–145

Yang WJ, Aida K, Terauchi A, Sonobe H, Nagasawa H (1996) Amino acid sequence of a peptide with molt-inhibiting activity from the kuruma prawn *Penaeus japonicus*. Peptides 17: 197–202

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