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Vitellogenin Production Induced by Eyestalk Ablation in Juvenile Giant Freshwater Prawn *Macrobrachium rosenbergii* and Trial Methyl Farnesoate Administration

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ABSTRACT—Juvenile *Macrobrachium rosenbergii* were bilaterally eyestalk-ablated and hemolymph-sampled at 3 to 6 day-intervals for three weeks. In males, vitellogenin appeared several days after ablation, increased slightly for 1–2 weeks, and then decreased. Quantification by enzyme immunoassay indicated peak vitellogenin levels ranging from 0.1 to 0.8 mg/ml. Females showed a similar profile, but peak levels ranged from 0.5 to 3.0 mg/ml; one individual was however exceptional with titers reaching nearly 30 mg/ml. Vitellogenin was not detectable in non-ablated animals. Juvenile male and female vitellogenin was shown by SDS-PAGE/Western blotting to consist of a single polypeptide component of 199K; however, in the exceptional female only, vitellogenin was composed of three polypeptides of 199, 102 and 90K as in adult female vitellogenin. Nevertheless, all ablated juvenile females exhibited increased gonadosomatic index and vitellogenic oocytes, as demonstrated by immunocytochemical techniques. Subsequently, juvenile males were employed to examine the effects of methyl farnesoate (MF) administration on vitellogenin production. In ablated animals, no significant differences in vitellogenin production were observed between the MF-injected and saline-injected groups. MF administration could not induce vitellogenin production in non-ablated animals. The physiological significance of the appearance of vitellogenin in juveniles of both sexes in context of the above results is discussed.

INTRODUCTION

In decapod crustaceans, the processes of molting and reproduction are inextricably linked; these are under the negative control of the peptide hormones, vitellogenesis-inhibiting hormone (VIH) and molt-inhibiting hormone (MIH). MIH and VIH originate in the X-organ/sinus gland complex of the eyestalk and have been extensively characterized in several species [5, 9, 29, 35]. Stimulatory factors also exist but their identities are not fully known; the thoracic ganglia have been demonstrated by several investigators [15, 33, 39] to be a source of a vitellogenesis-stimulating hormone (VSH) or gonad-stimulating hormone (GSH), and the ovary to be the putative site of vitellogenesis-stimulating ovarian hormone (VSOH) and factors considered to induce secondary sexual characteristics [20, 22, 32]. Also of much interest regarding crustacean reproduction, is the role of methyl farnesoate (MF), which in insects is the unepoxidated precursor of juvenile hormone (JH) III. MF, a product of the mandibular organs, has been detected in the hemolymph of adult *Macrobrachium rosenbergii* [27, 37] and has been demonstrated to be at high levels during active vitellogenesis in crabs [19].

It has been observed that eyestalk ablation results in the production of vitellogenin and the acceleration of ovarian maturation and spawning in adult female *Macrobrachium rosenbergii* [23], and in mature female Crustacea in general. However, little information has been available concerning the likelihood that juvenile crustaceans can synthesize vitellogenin and whether such factors as MIH and VIH are present at the juvenile stage. Therefore, the main aim of this investigation was to examine the effects of eyestalk ablation on vitellogenin production in juvenile *M. rosenbergii* of both sexes and to gain more understanding of what factors may be involved in the control of its production. The appearance of vitellogenin after eyestalk removal was followed, and the nature of juvenile vitellogenin was examined immunologically and electrophoretically.

Given the current implications that MF may be involved in vitellogenin synthesis in Crustacea as JH is in insects [21, 38], it was considered here that juvenile males could be used as a system for testing the effects of methyl farnesoate administration in *M. rosenbergii*. For trial purposes, MF was prepared from farnesoic acid and injected intramuscularly.

MATERIALS AND METHODS

Sampling: Vitellogenin production in ablated and non-ablated juveniles

Thirty juveniles (body weight, BW=2–5 g) were divided into two groups to be hemolymph-sampled for three weeks at intervals of several days irrespective of sex. In the first group (eyestalkless), bilateral ablation was carried out by simply holding the animal half-submerged in water, and snipping both eyestalks using scissors rinsed in crustacean saline. After allowing hemolymph flow to stop,

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the animal was fully returned to its tank. In the second group (intact) no treatment was employed. Hemolymph samples (less than 20 μl for each occasion) were taken by 25G needle and syringe, quick frozen at -80°C and stored at -30°C until analysis by enzyme immunoassay (EIA) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). At the end of the sampling period, animals were dissected for sex determination.

Enzyme immunoassay (EIA)

Antiserum raised against purified vitellin from *Macrobrachium nipponense* [14, 24] shown to be specific for vitellin in *M. rosenbergii* [23] was employed in EIA in this investigation. The details of the development and validation of this EIA have been reported previously [23]; this system utilizes *M. rosenbergii* vitellin for expression of the standard curve and *M. rosenbergii* hemolymph in the preparation of m-CB (0.01% male hemolymph-containing carbonate buffer used in the dilution of standards and samples). Otherwise, procedures are identical to those of the *M. nipponense* EIA [24] in which standards or samples are adsorbed onto wells, blocked, and subsequently incubated with vitellin antiserum followed by goat anti-rabbit IgG alkaline phosphatase conjugate.

In this investigation, after adding substrate (nitrophenylphosphate disodium salt) to wells, absorbance was measured at 405 nm; sample concentrations were calculated from the standard curve and expressed in terms of milligram equivalents of vitellin per milliliter hemolymph. The lower limit of detection was 0.03 mg/ml.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

SDS-PAGE was carried out on 7.5% polyacrylamide separating gels with 3% polyacrylamide stacking gels. Sample preparation was done by mixing sample dilution buffer (20 mM Tris-HCl pH 6.8, containing 4% SDS, 40% glycerol, 2% mercaptoethanol, and 0.005% bromophenol blue) and hemolymph diluted 10-fold with distilled water at a 1 : 1 ratio. These were heat-treated at 100°C for 5 min. For purposes of comparison, vitellin (Vn) purified by gel filtration and ion exchange chromatography from *M. rosenbergii* ovary [14] and female hemloymph from adult female *M. rosenbergii* with mature ovaries (reproductive molt) were also run concurrently. For molecular weight determination, high molecular weight (200, 116, 97, 66, 45K) markers (Bio-Rad molecular weight marker kit) were employed.

For subsequent immunoblotting procedures, proteins were firstly transferred electrophoretically from gels to Immunobilon PVDF Transfer Membranes (Millipore). Membranes were soaked in 10% Block Ace (Yukijirushi Nyugyou K.K.)/TBS (Tris-HCl pH 8.0 containing 0.9M NaCl), washed with TBS-Tween (TBS containing Tween 20) and transferred to a dilution of protein A purified and biotinylated anti-Vn IgG (approx. 3 μg/ml) in TBS-Tween. Membranes were stained using the Vectastain Avidin-Biotin Complex kit (Vector Laboratories) and 3,3′-diaminobenzidine (25 mg DAB, 4 μl 31% H_2O_2, 20 ml TBS-Tween). After saturating the membranes in DAB solution, staining was enhanced with the addition of 40 μl each 200 mM $CoCl_2$ and $NiCl_2$. Reference gels were stained with Coomassie brilliant blue.

Histology

Histological studies were employed to confirm the extent of vitellogenesis in ovaries of eyestalk-ablated juvenile females. Ovarian tissue was fixed for 24–48 hrs in Bouin's solution and dehydrated through an alcohol gradient as described previously [36]. Tissues embedded in paraffin were sectioned to 5–7 μm and stained with hematoxylin-eosin to examine ovarian stages.

Additionally, immunocytochemical techniques were undertaken to confirm the presence in oocytes of vitellin-immunoreactive material. Sections were treated with 3% hydrogen peroxide to block endogenous peroxidase activity, and incubated in anti-Vn IgG (Protein A purified) diluted to 1.3 μg/ml in 0.1 M phosphate-buffered saline (0.9% NaCl, pH 7.5) overnight at 4°C. Immunocytochemical procedures were done using the Histofine immunostaining kit (Nichirei): biotinylated goat anti-rabbit IgG was firstly applied to sections, followed by the application of peroxidase-conjugated streptavidin. Final staining was done using 3,3′-diaminobenzidine (12.5 mg DAB in 50 ml 0.1 M phosphate buffer, pH 7.4, containing 750 μl 0.3% H_2O_2). As a control, the same anti-Vn IgG dilution (1 ml) was preincubated overnight with 2 μg purified vitellin.

Methyl farnesoate (MF) injection experimental protocol

Forty juvenile males already showing development of male gonopores and petasma were chosen. These were divided into four groups to be subjected to the following protocol: 1) eyestalk ablation, MF injection; 2) eyestalk ablation, saline injection; 3) no ablation, MF injection; 4) no ablation, saline injection. MF was prepared from farnesoic acid (gift of Kuraray Co., Ltd.) by methylation with diazomethane; confirmation of identity was done via gas chromatography-mass spectrometry (GC-MS) as in Laufer *et al.* [19]. Stock solutions in ethanol at 4 mg/ml were made up and MF for injection was prepared as a suspension of 5% ethanol in crustacean saline. This gave 200 μg MF per ml and injections were carried out intramuscularly (at the base of the fifth pleiopod) using 25 μl, or 5 μg MF. In MF-injected animals, injections were carried out every day after ablation for 5 days, and then blood-sampled. In control animals, injection was done with 5% ethanol in saline. At the end of the experiment, experimental animals were dissected to confirm the presence of testes; hemolymph samples were taken and quick-frozen at -80°C and stored at -30°C until use in EIA and SDS-PAGE. Initial samples were taken in some cases to confirm non-detectable (ND) values at the outset. The Student's *t*-test was employed to examine final vitellogenin levels.

RESULTS

1. Vitellogenin production and molt frequency in juveniles

A. Enzyme immunoassay (EIA) and molting

In the first phase of this investigation, 20 individuals were ablated, and 10 were left intact and followed for a three-week period. Eyestalk ablation led to increased molting and rapid development of the gonads. Of 20 eyestalk-ablated individuals, 5 females and 6 males survived for the duration of the experiment. In addition, 2 females showing developed ovaries apparent through the carapace and 1 male showing gonopores survived into the second week. Other individuals did not survive after ablation. Of 10 intact individuals, 8 survived the three-week duration; animals were dissected, but sex could not be determined. Ovaries of eyestalk-ablated females were additionally examined histologically (see below).

Vitellogenin titers of individuals followed for the three week duration are shown for males M1–6 (Fig. 1), females F1–5 (Fig. 2a, b) and intact animals, L1–8 (Fig. 3). In males

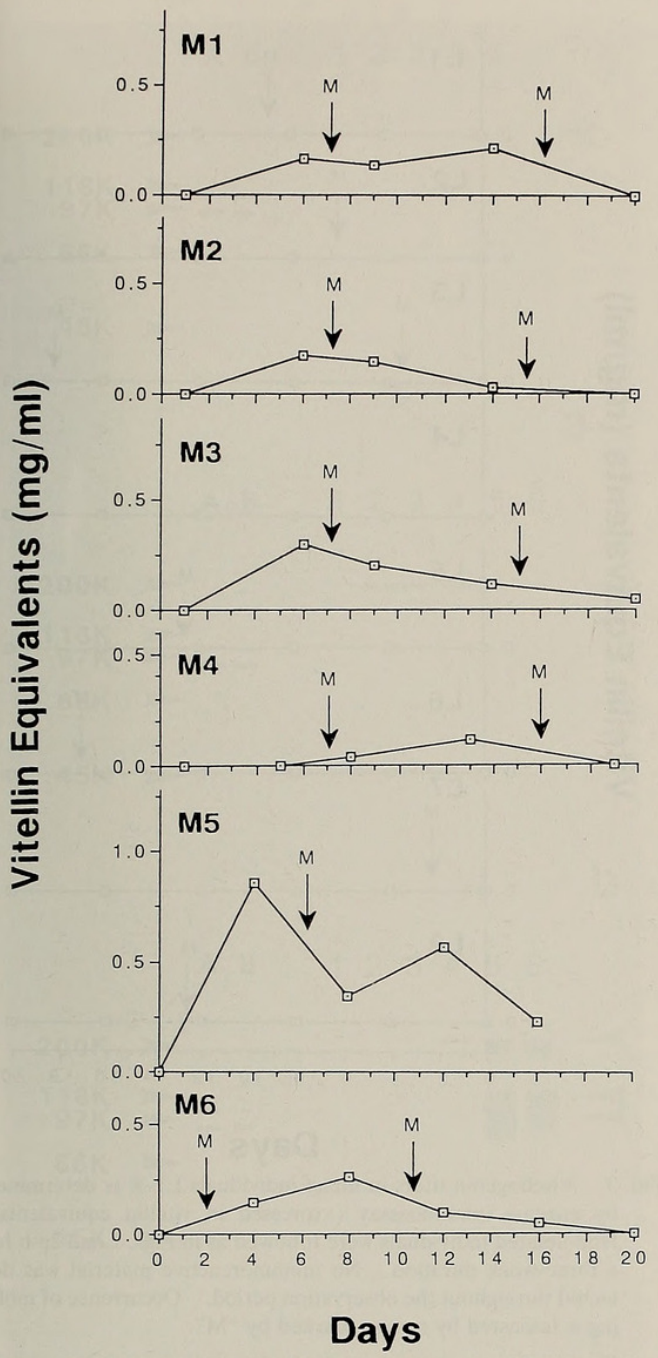


FIG. 1. Vitellogenin titers in ablated juvenile males M1–6 as quantified by enzyme immunoassay (expressed in vitellin equivalents). Peak levels ranged from 0.1 to 0.8 mg/ml. Vitellogenin appeared several days after ablation, increased slightly for 1–2 weeks, and then decreased. Eyestalk removal was performed on Day 0, and the first hemolymph sample was taken several hours later or within 24 hr of this. Individuals were then followed for the days indicated. Arrows marked by "M" indicate occurrence of molting.

(body weight, BW = 4.02 ± 0.45 g), no immunoreactive material was detectable at initial sampling occasions, performed within 24 hr of ablation. Vitellogenin generally appeared several days after ablation, increased slightly for 1–2 weeks, and then decreased. Molts are indicated by arrows; all but one male individual molted twice during the twenty-day period. Maximum titers were between 0.1 and 0.8 mg/ml.

Females (BW = 3.80 ± 0.29 g) also molted twice during the 3-week duration, but peak levels were greater than those of males, ranging between 0.5 to 3.0 mg/ml. F5 was however exceptional, with levels reaching almost 30 mg/ml. It should be noted that in this species, normally maturing adult females exhibit peak Vg levels around 10 mg/ml [23]. Intact animals (BW = 3.10 ± 0.18 g) were non-detectable (ND) throughout the experiment and molted 0–1 times.

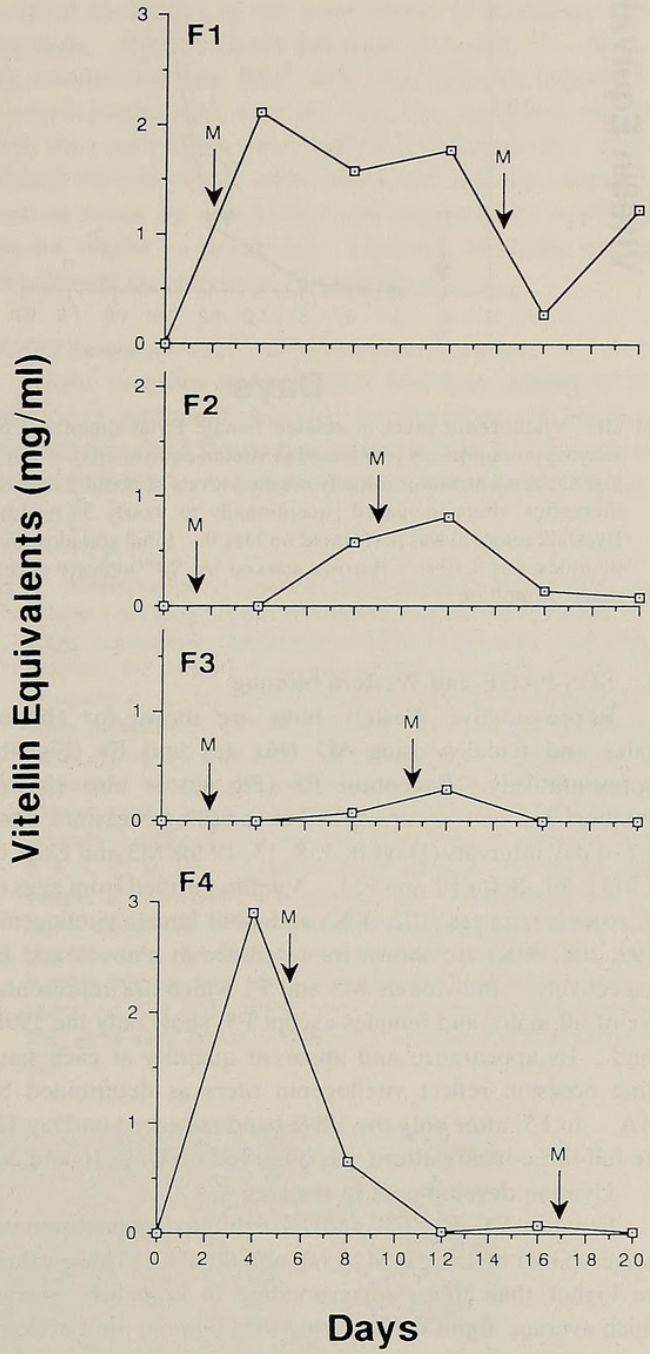


FIG. 2a. Vitellogenin titers in ablated juvenile females F1–F4 as quantified by enzyme immunoassay (expressed in vitellin equivalents). Peak levels ranged from 0.5 to 3 mg/ml. Vitellogenin appeared several days after ablation, increased slightly for 1–2 weeks, and then decreased. Eyestalk removal was performed on Day 0, and the first hemolymph sample was taken several hours later. Individuals were then followed for the days indicated. Gonadosomatic index ranged from 0.54 to 1.02%. Arrows marked by "M" indicate occurrence of molting.

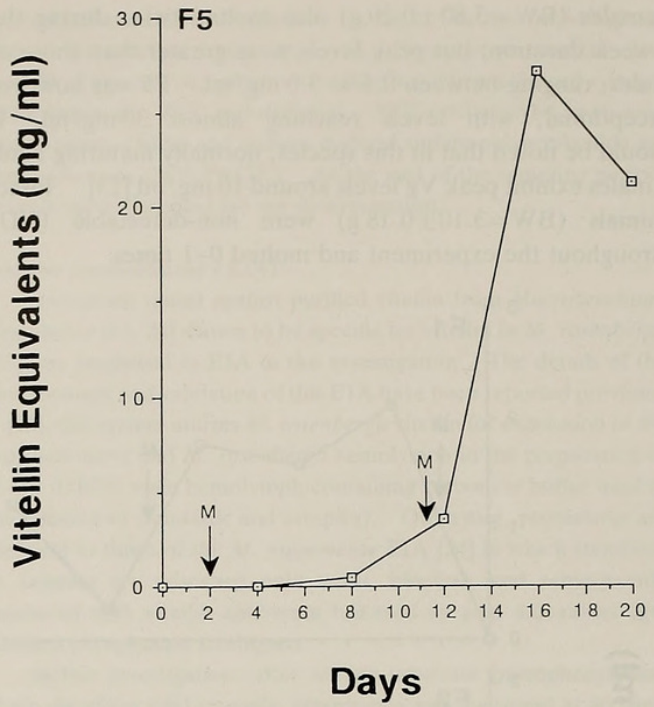


FIG. 2b. Vitellogenin titers in ablated female F5 as quantified by enzyme immunoassay (expressed in vitellin equivalents). Vitellogenin concentrations initially reached levels of about 3 mg/ml; thereafter, titers increased exceptionally to nearly 30 mg/ml. Eyestalk removal was performed on Day 0. Final gonadosomatic index was 4.10%. Arrows marked by "M" indicate occurrence of molting.

B. SDS-PAGE and Western blotting

Representative Western blots are shown for ablated males and females using M3 (Fig. 4a) and F1 (Fig. 4b) representatively. Exception F5 (Fig. 4c) is also shown. Numbers 1–5 or 1–6 correspond to sampling occasions done at 3–6 day intervals (Days 0, 3, 8, 13, 19 for M3 and Days 0, 4, 8, 12, 16, 20 for F1 and F5). Vitellin purified from eggs of *M. rosenbergii* eggs (102, 90K) and adult female vitellogenin (199, 102, 90K) are shown for reference in lanes A and B, respectively. Individuals M3 and F1 which are representative of all males and females except F5, show only the 199K band. Its appearance and apparent quantity at each sampling occasion reflect vitellogenin titers as determined by EIA. In F5, after only the 199K band appeared on Day 12, the full three-band pattern was observed on Days 16 and 20.

C. Ovarian development in females

Females F1, F2, F3, and F4 exhibited gonadosomatic indices (GSI) of 1.02, 0.54, 0.60, and 0.65%. These values are higher than those corresponding to immature ovaries which average about 0.4% during the common molt cycle of the adult female, but are low compared to GSI values that

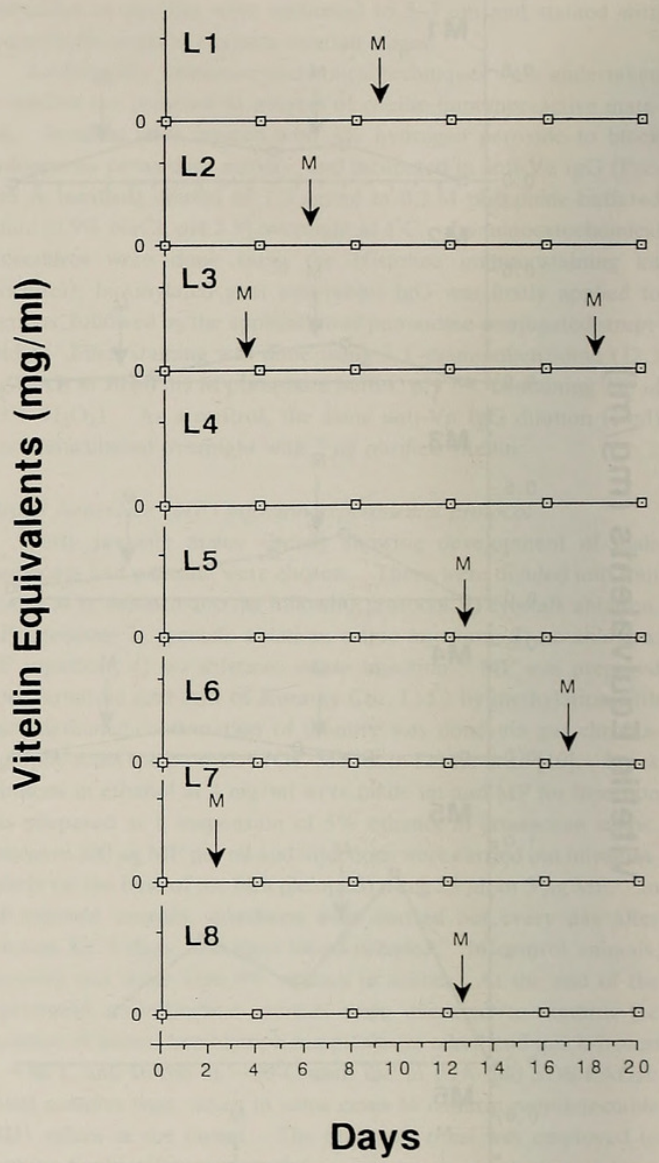
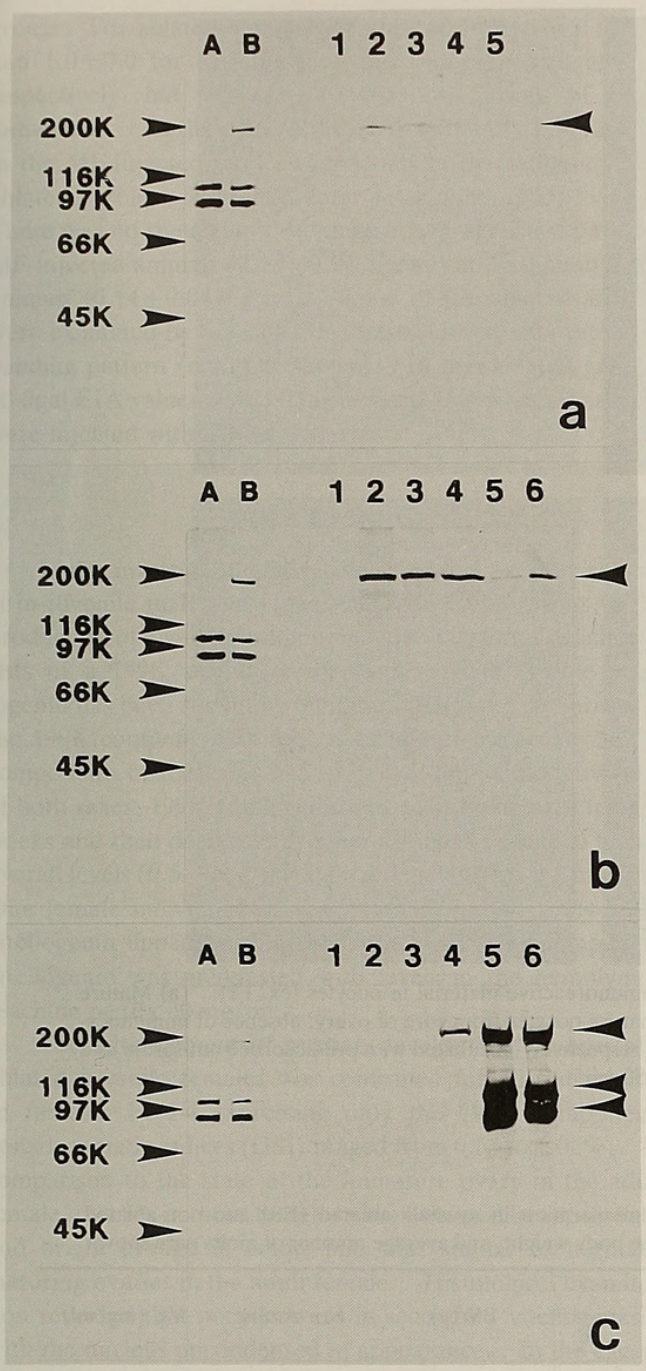


FIG. 3. Vitellogenin titers in intact individuals L1–8 as determined by enzyme immunoassay (expressed in vitellin equivalents). Non-ablated individuals were followed as in Figs. 1 and 2a-b for a three-week duration. No immunoreactive material was detected throughout the observation period. Occurrence of molting is indicated by arrows marked by "M".

reach above 9 percent in fully mature ovaries [36]. F5 with exceptionally high vitellogenin levels also had a much greater GSI, of 4.10%. In histological examination using hematoxylin-eosin, it was observed that ovaries in females F1–F4 were in secondary vitellogenesis, exhibiting oocytes partially filled with yolk globules. In these oocytes, the nucleoli were clearly visible within the nucleus, which was not condensed in appearance (Fig. 5a). Ovaries in female F5 were quite

FIG. 5. Extent of oocyte development in ablated juvenile females. (a) F1 was typical of the degree of ovarian maturity in females F1–F4. Oocytes are enlarged, contain yolk globules, and can be considered to be in secondary vitellogenesis. The nucleoli are clearly visible within the nucleus. Germinal vesicle breakdown (GVBD) has not yet begun, as the nucleus is not condensed in appearance. Gonadosomatic index (GSI) ranged from 0.54 to 1.02%. (b) F5, exhibiting extremely high titers of full vitellogenin, possessed oocytes which appeared to have undergone GVBD as the nucleus is no longer observable. However, F5 did not undergo spawning. GSI was above 4%. Scale bar: 50 μm.



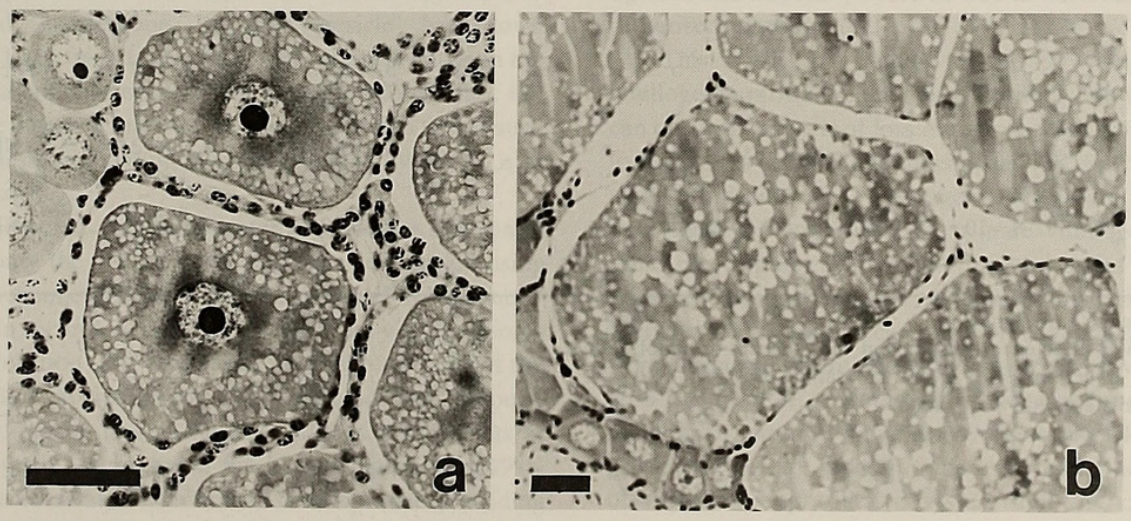
different in appearance (Fig. 5b). Oocytes were extremely enlarged in size in comparison to those of the other individuals. The nucleus was not present, and oocytes gave the appearance of having undergone germinal vesicle breakdown (GVBD) [36]. However, this individual did not spawn eggs within the duration of the experiment.

Employing immunocytochemical techniques, we were able to confirm the accumulation of vitellin-immunoreactive material in oocytes in the latter stages of secondary vitellogenesis. Figure 6 shows histology for female F1. Maturing oocytes partially filled with yolk globules indicate an immunocytochemical reaction (a), but immature oocytes from the center of the ovary exhibit no reaction (b). In the control version of the same individual, mature oocytes in sections which were incubated with pre-absorbed antibodies did not exhibit a reaction (c). Likewise, immature oocytes also showed no indication of staining (d).

Methyl farnesoate (*MF*) administration in males

Male juveniles were divided into four groups of ten individuals each and received combinations of ablation/non-ablation and MF injection/saline injection. Results are shown in Table 1, along with average body weights for each

FIG. 4. Examination of juvenile vitellogenin by SDS-PAGE and Western blotting. (a) Vitellogenin was shown to consist of a single polypeptide component of 199K in juvenile males (M1–M6) (ex. M3; lanes 1–5 correspond to Days 0, 5, 8, 13, 19). In comparison, adult female vitellogenin (lane B) was composed of three polypeptides of 199, 102, and 90K, and purified vitellin (lane A) of 102 and 90K. Western blotting results were in agreement with enzyme immunoassay results. (b) Vitellogenin consisted of a single polypeptide component of 199K also in juvenile females (F1–F4) (ex. F1; lanes 1–6 correspond to Days 0, 4, 8, 12, 16, 20). Lanes A and B are as in (a). (c) Juvenile female F5, however, was exceptional. Vitellogenin first became detectable by enzyme immunoassay (lane 4–Day 12), showing only the 199K component, but thereafter, full vitellogenin became very abundant, and all three peptide components, e.g. 199, 102 and 90K, were observed (lanes 5, 6–Days 16, 20), as in adult female vitellogenin. Lanes A and B are as in (a).



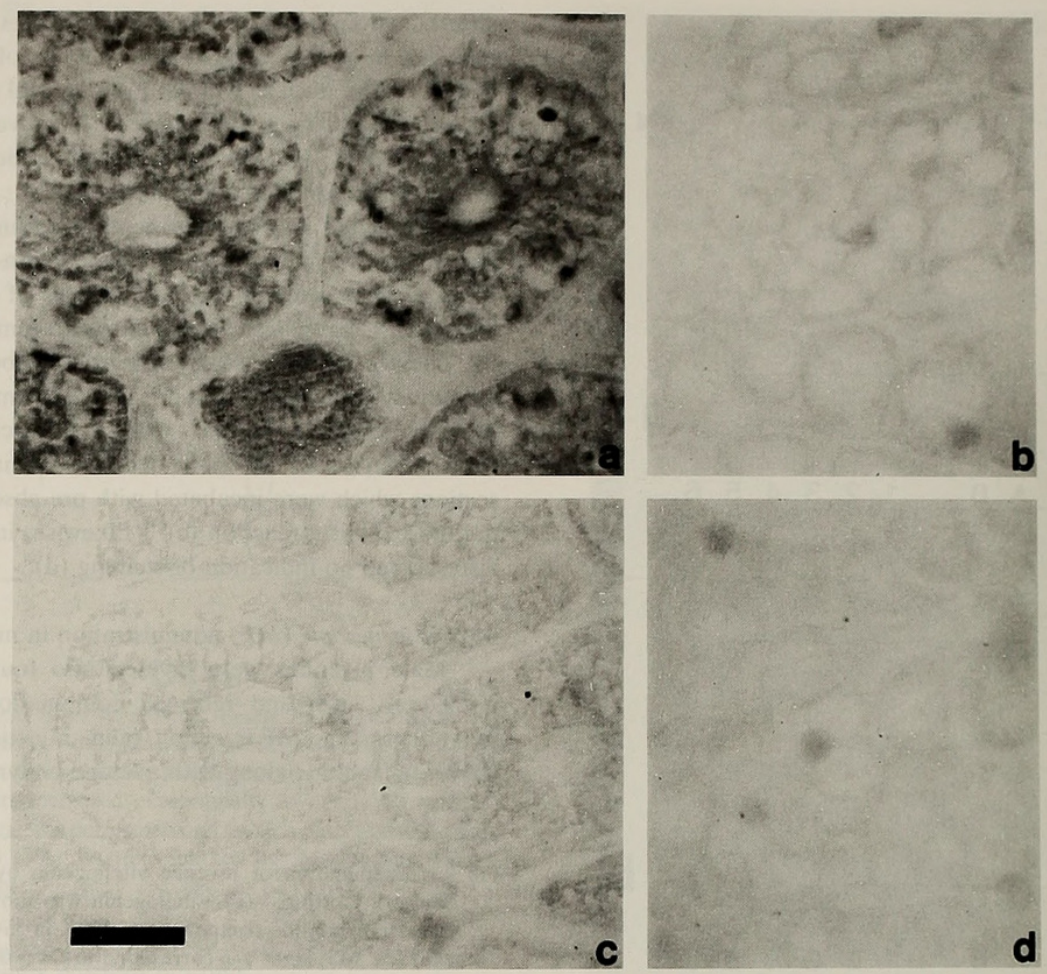


FIG. 6. Immunohistology, and examination of uptake of vitellin-immunoreactive material in oocytes (ex. F1). (a) Mature oocytes, occurrence of immunocytochemical reaction. (b) Immature oocytes from core of ovary; absence of immunocytochemical reaction. (c) and (d) Same oocytes as in (a) and (b) respectively, incubated with pre-absorbed antiserum as a control; absence of immunocytochemical reaction. Scale bar: 50 μm.

TABLE 1. Experimental protocol for methyl farnesoate (MF) administration in eyestalk-ablated (ES) and non-ablated (intact) juvenile males. Data for final vitellogenin levels, average body weight, and average number of molts pare shown

Treatment	No. ind.	Schedule (Days)							BW(g)	No. molts	Vg (mg/ml)
		0	1	2	3	4	5	6			
MF inj. + ES	N=10	a	*	*	*	*	*	h	3.24±0.38	0.6±0.2	0.15±0.04[1]
Saline + ES	N=10	a	o	o	o	o	o	h	3.82±0.11	1.0±0.0	0.14±0.04[1]
MF inj. + Intact	N=10		*	*	*	*	*	h	3.21±0.21	0.3±0.2	ND
Saline + Intact	N=10		o	o	o	o	o	h	2.37±0.24	0.3±0.2	ND

[1] Difference not significant.
* 5 μg MF injected as suspension in 25 μl 5% ethanol/saline.
o Blank injection as 25 μl ethanol saline.
a Eyestalk ablation performed.
h Hemolymph samples taken.

group. For ablated animals, number of molts was 0.6 ± 0.2 and 1.0 ± 0.0 for MF-injected, and saline-injected groups, respectively, but was 0.3 ± 0.2 for both groups of intact individuals. At the end of the experiment, only 3 individuals in the MF-injected, and 2 individuals in the saline-injected ablated groups exhibited non-detectable (ND) values. There was no significant difference in average value between MF-injected animals (0.15 ± 0.04 mg/ml) and saline-injected animals (0.14 ± 0.04 mg/ml). Some of the ablated animals were examined by SDS-PAGE; these showed only the 199K banding pattern (data not shown). In non-ablated groups, all final EIA values were ND, irrespective of whether animals were injected with MF or with saline.

DISCUSSION

In this investigation, it was revealed that eyestalk removal in juvenile male and female *M. rosenbergii* results in the production of a vitellin-immunoreactive substance which consists of a 199K peptide component. Adult female vitellogenin has been shown by Okumura [23] to be comprised of the 199K component as well as of lighter molecular weight components of 102K and 90K. In eyestalk-ablated juveniles of both sexes, 199K vitellogenin was seen to increase for 1–2 weeks and then decrease; in general, females reached higher overall levels (0.5–3 mg/ml) than did males (0.1–0.8 mg/ml). One female individual was exceptional; initially only 199K vitellogenin appeared, but thereafter, full three-component vitellogenin was manifested with levels in the hemolymph reaching nearly 30 mg/ml.

The advancement of ovarian development in eyestalk-ablated juvenile females was confirmed for all individuals. In females F1–F4 expressing only the 199K component, gonadosomatic indices (GSI) ranged from 0.54 to 1.02%. In comparison to the state of the immature ovary in the adult female, ovaries in these females were enlarged in appearance and bright orange in color, and thus similar to normally maturing ovaries in the adult female. Histological examination revealed that oocytes were in secondary vitellogenesis, with the nucleus uncondensed in appearance. In the exceptional female F5, GSI was 4.10% and oocytes appeared to have already undergone germinal vesicle breakdown (GVBD). These results suggested a correlation between the extent of ovarian development and the ability to produce full vitellogenin; whether this is related to an ovarian factor such as vitellogenesis-stimulating ovarian hormone (VSOH), is as of yet unclear.

The accumulation of yolk proteins in oocytes of females expressing only the 199K band was further confirmed using immunocytochemical techniques. In females F1–F4, maturing oocytes in secondary vitellogenesis stained via immunocytochemical reaction, and immature oocytes in the core of the ovary exhibited no reaction. This indicated that although juvenile vitellogenin lacked the 102 and 90K components of adult vitellogenin, increases in the hemolymph of the 199K peptide were correlated with oocyte development, providing further support that juveniles, not only females, but also males have the ability to produce vitellogenin to a certain extent. The small size of the ovaries of the above individuals did not permit analysis on SDS-PAGE in parallel to the above histological studies; this will be investigated subsequently.

While little information is available concerning vitellogenin in juveniles, vitellogenin and vitellin structure have been examined in a number of adult crustacean species [8, 25, 26, 34]. Adult *M. rosenbergii* seems similar to many of these; for example, in *Penaeus monodon,* vitellogenin and vitellin are comprised of subunits of 74, 83, 104 and 168K [26]. Less is known however, concerning how crustacean vitellogenins and vitellins are processed while becoming sequestered in eggs. In the terrestrial isopod, *Armadillidium vulgare* [30], four forms of vitellin are initially accumulated in oocytes, but the higher molecular weight ones undergo proteolytic processing, leaving only the lightest component. Komatsu and Ando [18] have reported a low density lipoprotein (LDL) present in the egg yolk of the sand crayfish, *Ibacus ciliatus,* which degrades vitellogenin and may be involved in vitellogenin processing in this species.

The native form of vitellin in *M. nipponense* has been estimated by gel filtration as 350K [14], and preliminary work indicates that this is similar in *M. rosenbergii* (Okumura, unpublished data). In *M. rosenbergii,* as vitellin yields the 102K and 90K components on SDS-PAGE, it seems plausible that the 199K protein is initially synthesized as a precursor vitellogenin and undergoes further processing in the mature female in relation to a factor perhaps produced by the ovary. Vitellin in its final form may be a multimer association of the 102K and 90K proteins subunits, thus the 199K protein would not appear in eggs, but occurs in the hemolymph. Of note, in Derelle *et al.* [12], *M. rosenbergii* female vitellogenin and vitellin examined on SDS-PAGE was comprised of subunits of 84 and 92.2K. Regarding vitellin, these results are similar to those obtained in our investigation. However, these authors did not observe a higher molecular weight component in hemolymph vitellogenin, such as the 199K component obtained here. Discrepancies in results between their investigation and this study could be due to differences in methods of antisera preparation.

This is the first report to our knowledge concerning vitellogenin production in male decapods, but in isopods, this can occur as a result of loss of androgenic gland function due to natural or artificial circumstances [28, 31], In *A. vulgare* andrectomized males, vitellogenin levels are in fact higher than those of normal females; male vitellogenin does not differ electrophoretically from that of females [31], although the ovary is suggested to be necessary to maintain vitellogenin titers during the molt cycle. In *Porcellio dilatatus,* fat bodies from surgically-untreated males synthesize vitellogenin *in vitro* [13]. In *M. rosenbergii,* vitellogenin production can be induced by simply removing the eyestalks. As some sinus gland peptides are thought to be involved in the maintenance of the male reproductive system and the control of the

androgenic gland in other decapod species [3, 10], in this investigation, whether the appearance of vitellogenin in *M. rosenbergii* is due to the absence of a putative VIH or is more related to the removal of other eyestalk factors, is at present unclear. However, vitellogenin levels in males do not reach those of juvenile females, suggesting that there may be additional factors of female origin involved in further stimulation of early vitellogenin production. On the other hand, in adult males, eyestalk ablation results in the appearance of vitellogenin in only occasional individuals (Yang *et al.*, unpublished data); this suggests some involvement of the androgenic gland in that it is expected to be less developed and therefore less inhibitory in juveniles than in adults.

In male insects, several authors have studied by immunological and electrophoretic methods the induction of vitellogenin synthesis by juvenile hormone and/or ecdysone treatment [2, 16, 21]. The actions of juvenoids and ecdysteroids vary with species. Agui *et al.* [4] have examined production of vitellogenin mRNA in male and female housefly *Musca domestica,* revealing that in males, 20-hydroxyecdysone, but not juvenile hormone can stimulate transcription of the vitellogenin genes; this has indicated the necessity of the ovary. In *Diploptera punctata,* vitellogenin synthesis is directly inducible by application of juvenile hormone analogue [21]. Induced male vitellogenin levels are often lower than levels in normal females (similar to *M. rosenbergii*).

Along these lines, it was postulated that methyl farnesoate (MF) may be necessary for further stimulation and processing of vitellogenin in *M. rosenbergii.* MF being highly insoluble in water, was firstly dissolved in stock solutions of ethanol, which were adjusted in crustacean saline. This formed a cloudy suspension. However, as discussed in the results, MF injection did not produce significantly higher levels of vitellogenin (0.15 ± 0.04 mg/ml) above those of ablated animals injected with saline (0.14 ± 0.04 mg/ml). MF injection also did not induce vitellogenin synthesis in non-ablated animals. In preliminary work, other methods of injection were attempted, for example, cardiac injection with either MF (and farnesoic acid) in saline or in purified sesame oil, or similar intramuscular injections using oil instead, but these were also ineffective in stimulating vitellogenin production in either sex. Thus, eyestalk removal was observed to be a prerequisite for induction of vitellogenin production in males, but MF administration seemed to have no influence.

From these results, it was not possible to obtain positive evidence that MF has any connection to vitellogenin production in *M. rosenbergii,* but it also can not be conclusively stated that MF has no physiological role whatsoever. MF has been shown to activate Na/K-ATPase in *Artemia,* indicating its potential role in osmoregulation and molting [6, 7]. MF has been detected in both sexes in *M. rosenbergii* by Sagi *et al.* [27]; we have additionally determined that MF is present in females during both the reproductive molt and common molt cycles, and seems highest during the early premolt stages [37]. Additionally, it is not ruled out that MF has no involvement in the process of vitellogenin uptake. In insects, juvenoids affect increased membrane Na/K-ATPase activity in the ovarian follicles, causing cell volume to shrink, and creating spaces through which vitellogenin can pass through to access the oocytes [1, 11, 17]. This is known as "patency".

The results of this study have suggested that vitellogenesis- and molt-inhibiting eyestalk factors are present in juveniles as well, but this is not sufficient to explain the means by which precursor vitellogenin is processed into the full vitellogenin observed in the adult female and how vitellogenin production is possible in males. In subsequent studies, it will be important to investigate the involvement of ovarian factors in concert with MF and the relationship between androgenic gland functioning and male vitellogenin production.

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