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# Induction of vitellogenesis by 17-hydroxyprogesterone and methyl farnesoate during post-reproductive period, in the estuarine crab *Neohelice granulata*

Daniel A. Medesani[a,b], Lilian E. Ferré[a], Ivana S. Canosa[a,b], Gabriela R. Silveyra[a,b] and Enrique M. Rodríguez[a,b]*

[a]*Department of Biodiversity and Experimental Biology, Faculty of Exact and Natural Sciences, University of Buenos Aires, Buenos Aires, Argentina;* [b]*Institute of Biodiversity, Experimental and Applied Biology (IBBEA, CONICET-University of Buenos Aires), Buenos Aires, Argentina*

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Adult female crabs (*Neohelice granulata*) were treated during the post-reproductive period with both 17-hydroxyprogesterone (17PG) and methyl farnesoate (MF). During the 4-week *in vivo* assay, animals were fed hormone-enriched (17PG or MF) pellets twice a week, at a dose of 2 nmol/g of body weight. Additionally, at the middle of the *in vivo* experiment, ovarian explants were incubated *in vitro* with each hormone at a concentration of 15 μM for 24 h. At the end of the *in vivo* assay, both the gonadosomatic index and the vitellogenic protein content (Vg) of crabs treated with either 17PG or MF were significantly higher ($p < 0.05$) than the values of the concurrent controls. In addition, the hemolymphatic vitellogenin level of both hormonally treated groups was higher. At the end of the *in vitro* assay, ovary explants incubated with 17PG had a significantly higher ($p < 0.05$) protein synthesis and content of Vg proteins than controls. Ovary explants incubated with MF had similar levels to controls ($p > 0.05$). The involvement of these hormones in the regulation of ovarian growth during the reproductive cycle of *N. granulata* is discussed.

**Keywords:** crabs; ovary; reproduction; 17-hydroxyprogesterone; methyl farnesoate



## Introduction

*Neohelice* (=*Chasmagnathus*) *granulata* is a crab species widely distributed along estuarine coasts of both Argentina and Brazil (Boschi 1964). A marked seasonality characterizes its reproductive cycle, i.e. adult females spawn only during spring and summer. During the post-reproductive period (autumn), the ovary is quiescent; however, a significant proportion of adult females continue to molt during this period (López Greco & Rodríguez 1999; Stella et al. 2000). *N. granulata* is an important species because several fish species feed on it and because it serves as a model species for several physiological and ecological research lines (Menni 1983; Sánchez et al. 1991). For example, its endocrine control of reproduction has been previously studied to some extent (Medesani et al. 2004; Rodríguez et al. 2007).

Both vitellogenin and vitellin are high molecular weight lipoproteins that are the main components of vitellum, which is essential for embryonic development. In most decapod crustaceans, vitellogenin is synthesized by the hepatopancreas, secreted to the hemolymph, and taken up by oocytes to be used as the precursor of vitellins (Tsukimura 2001). In addition to this exogenous vitellogenesis, an endogenous synthesis of vitellins takes place in the ovary throughout the ovarian maturation (Charniaux-Cotton & Payen 1988). Vitellogenesis is under hormonal control; to this respect, several environmental factors (such as photoperiod and temperature) trigger the secretion of neurohormones by the thoracic ganglion, brain, and the X organ–sinus gland complex located in the eyestalks (Fingerman 1997). These neurohormones in turn regulate the secretion of two hormone families of non-peptidic nature; one of them is represented by vertebrate-like steroids such as 17-hydroxyprogesterone (17PG), and the other by sesquiterpenoids, mainly methyl farnesoate (MF), also known as the juvenile crustacean hormone (Fingerman 1997; Rodríguez et al. 2007).

The ability to synthesize vertebrate-like steroids, such as progesterone and estradiol, has been reported in either gonads or hepatopancreas of several crustaceans species (reviewed by Lafont & Mathieu 2007, and previously by Fingerman et al. 1993). Additionally, the ability of some crustacean tissues, such as ovary, hepatopancreas, gill, and abdominal muscle, to metabolize such steroids has been reported (Young et al. 1992). Occurrence of both progesterone and estradiol has been also reported in hemolymph, hepatopancreas, and gonads of several crustacean species (Quinitio et al. 1994; Shih & Tseng 1999; Warrier et al. 2001). Females shrimp (*Penaeus japonicas*) injected with 17PG increased their serum levels of vitellogenin (Yano 1987). *In vivo* studies of *Cherax albidus* by Coccia et al. (2010) showed that both estradiol and 17PG produce a higher synthesis of vitellogenin in

*Corresponding author. Email: enrique@bg.fcen.uba.ar

the hepatopancreas and/or the hemolymph, depending on the reproductive cycle period. A direct effect of 17PG on oocyte growth was seen *in vitro*, in two crab species: *N. granulata* (Zapata et al. 2003) and *Oziotelphusa senex senex* (Reddy et al. 2006).

MF, secreted by the mandibular organ of crustaceans, is a multifunctional hormone, involved in processes such as molting, reproduction, larval development, and morphogenesis (Homola & Chang 1997). This hormone is closely related both chemically and physiologically to the insect juvenile hormone III, as it is involved in both exogenous vitellogenesis and vitellogenin uptake (Laufer et al. 1992; Nagaraju 2011). Recent studies in the crayfish *Cherax quadricarinatus* also indicate that MF may have a direct effect on the synthesis of ovarian proteins, i.e. endogenous vitellogenesis (Medesani et al. 2012). The *in vivo* administration of MF to the crayfish *Procambarus clarkii* stimulated ovarian growth in terms of an increase in both the gonadosomatic index (GSI) and the oocyte diameter (Laufer et al. 1998; Rodríguez et al. 2002a).

This study is aimed at evaluating the effect of 17PG and MF, both *in vivo* and *in vitro*, on the concentration of vitellogenic proteins (vitellogenin and vitellins) in the ovary, hepatopancreas, and hemolymph of *N. granulata* mature females.

## Materials and methods

### In vivo *assay*

This assay was conducted during the post-reproductive period for four weeks (mid May to mid June). Adult females weighing 11.03 ± 0.15 g ($N = 45$) were used. Controlled conditions of both photoperiod (12:12 L:D) and temperature (23 ± 1 °C) were maintained throughout the experiment and during the 7-day acclimation period, prior to the start of the experiment. Fifteen animals were assigned to each of the three treatments: 17PG, MF, or control. For each treatment, three plastic containers filled with 2 L of saline water at 12 g/L, prepared with dechlorinated water and commercial salts (Tetra Marine Salt Pro®), were used, placing five animals in each recipient. To serve as refuges, small PVC pipes were provided. Water was changed twice a week.

Pellet food containing 33% protein, 9% lipids, and 26% carbohydrates was prepared using fish and soybean flours, the main protein sources, according to a previous study (Gutiérrez & Rodríguez 2010). Pellets weighing 0.12–0.14 g each were prepared and dried at 40 °C for 24 h in order to make them stable in water for at least 3 h. Some of these pellets were enriched with hormone by adding 17PG or MF previously dissolved in ethanol, in such quantity that consumption of a single pellet by each animal represented a dose of 2 nmol/g of body weight. Control pellets had only ethanol added.

During the assay, all animals were fed daily at a rate of 2% biomass/day (according to Lawrence & Jones 2002). Twice a week, each animal in the 17PG or MF experimental group was fed one hormonally enriched pellet, and in the remaining days of the week, crabs were fed control pellets. Additionally, three times a week, all animals were fed fresh *Elodea* sp. as a dietary supplement. All containers were monitored daily to detect molting or mortality. Both the experimental protocol and the dose of hormones used were similar to those used in previous studies (Rodríguez et al. 2002a, 2002b; Zapata et al. 2003).

At the end of the *in vivo* assay, both the gonadosomatic (GSI) and hepatosomatic (HSI) indexes were calculated by computing the ratio of mass of the ovary or hepatopancreas to body weight, respectively. Samples of both the ovaries and the hepatopancreas were also taken to determine the level of vitellogenic proteins (Vg), according to the protocols specified below. In addition, GSI, HSI, and Vg levels in both the ovaries and the hepatopancreas were determined in a group of control animals at the beginning of the experiment to establish an initial baseline. In addition, samples of hemolymph from all animals were taken at the start, middle (2nd week), and end (4th week) of the assay.

#### *Processing of ovaries for vitellogenic proteins determination*

Ovaries were cut into small explants (0.1–0.2 g), which were homogenized in sodium phosphate buffer (50 mM, pH 7.4, with 2 µL/mL of protease inhibitor), in a 1:3 (w/v) ratio. Each homogenate was then centrifuged at 10,000 g for 20 min at 4 °C. Supernatants were further ultra-centrifuged (100,000 g for 50 min, at 4 °C); the resulting supernatants were stored in Eppendorf tubes and frozen at −70 °C until analysis by ELISA.

#### *Determination of vitellogenic protein levels*

The ELISA procedure previously employed by Ferré et al. (2012) was used. Briefly, a primary antibody against Vg (vitellins and vitellogenin, both present in immature ovaries) was obtained by inoculating rabbits with purified Vg, according to previous studies (Dreon et al. 2003; García et al. 2008). Peroxidase-conjugated anti-IgG from the rabbits (Bio-Rad Lab) was used as the secondary antibody. Purified Vg in a 1/500 dilution was used to prepare the standard curve. Triplicate 50-µL aliquots of either the standard or the sample were placed in a 96-well plate (Nunc-Immunoplate Polisorp). Samples were previously diluted in a coating buffer (15 mM sodium carbonate, 35 mM sodium bicarbonate, pH 9.6). Both primary and secondary antibodies were diluted (1/500) in PBS + 0.05%, Tween, and 6% powdered milk. Absorbance was measured in plate reader at 415 nm, (Bio-Rad

Lab., Model 680). 2-20-Azino-di-3-ethylbenzthiazoline sulfonic acid was used as the chromogen.

The GSI and HSI indexes, as well as Vg levels, were analyzed using one-way ANOVA, followed by planned comparisons, once the assumptions for this test were confirmed. Both molting and mortality percentages were compared among treatments (as proportions) by the Fisher's exact test (Sokal & Rohlf 1981). A 5% confidence level was always used.

### In vitro *assays*

At early June, stock female crabs (maintained under the same conditions used for controls of the *in vivo* assay) were used for the *in vitro* experiments. Ovarian explants (approximately 1 × 0.5 cm each) were incubated for 24 h in $CO_2$ chambers at 27 °C and in constant darkness. Each female provided a similar piece of ovary to one vial of every group of the same experiment (repeated measure design). Medium 199 was prepared using powdered medium with L-glutamine and Earle's salts (Sigma Chemical Co.), dissolved in crustacean saline (Cooke et al. 1977), and modified to compensate for the salts already present in the culture medium. In addition, as in previous studies (Sarojini et al. 1997; Rodríguez et al. 2000), 6 mg of penicillin-G per 100 mL of medium was added to prevent bacterial growth, and the pH was adjusted to 7.4 with 0.5 N NaOH.

Each vial, filled with 2 mL of culture medium, received an aliquot of 20 µL from the stock solution of any of the hormones assayed, to achieve a hormonal concentration of 15 µM. Each vial of control group received an aliquot of 20 µL of ethanol, used as the vehicle for either hormone. 17PG was acquired from Sigma Chemical Co. (St. Louis, Mo), whereas MF (trans-trans) was synthesized from commercial geranylacetone, according to Rodríguez and Gros (1990).

#### *Protein synthesis assay*

Tritiated leucine was added to the culture medium to estimate ovarian protein synthesis by its incorporation to the acid precipitable protein fraction following the methodology previously used for *N. granulata* and other crustacean species (Eastman-Reks & Fingerman 1984; Rodríguez et al. 2002a, 2002b; Zapata et al. 2003; Medesani et al. 2004). Briefly, an aliquot of 30 µL from the $^3$H-leucine stock solution (1:10 dilution from 1 mCi/mL, NEN Life Science, Inc.) was added to each vial, to reach a total activity of 3 µCi. At the end of the incubation period, the ovarian explants were weighed and homogenized in 2 mL cold 10% trichloroacetic acid (TCA) followed by a centrifugation at 5000× *g* for 10 min at 4 °C, then washed twice with cold TCA, resuspended, and decanted into a Millipore suction filtration funnel using 0.22-µm nitrocellulose filter disks. After filtration, the disks were air-dried for 1 h and submerged in scintillation fluor solution (Optiphase Hi Safe 2) overnight at 4 °C. Radioactivity was measured in a Beckman scintillation counter. Uptake of labeled leucine by the ovary was expressed on ovarian wet weight basis (CPM/mg ovary).

#### *Vitellogenic proteins concentration assay*

An additional assay, with the same experimental groups used for the protein synthesis assay, was performed to determine the total content of vitellogenic proteins (vitellogenin and vitellins) at the end of the 24 h incubation period. To determine the content of these proteins, the same methodology described for the *in vivo* assay was followed.

Results of both the *in vitro* assays were analyzed using one-way repeated measures ANOVA, followed by planned comparisons (Sokal & Rohlf 1981).

## Results

### In vivo *assay*

Table 1 shows the mean weight of females at the start and end of the assay, as well as molting and mortality percentages. No significant differences ($p > 0.05$) among the treatments were detected in any case. However, both experimental groups showed a significantly higher GSI ($p < 0.05$) than that of the control group (Figure 1). Correspondingly, the Vg protein content in the ovary of female crabs treated with either 17PG or MF was also significantly higher ($p < 0.05$) compared to the concurrent control (Figure 1). No significant differences in GSI or Vg protein content ($p > 0.05$) were seen between the initial and concurrent control.

Table 1. Mean body weight (± standard error) of females used for the *in vivo* assay.

| Experimental group | Initial body weight (g) | Final body weight (g) | % Molting | % Mortality |
|---|---|---|---|---|
| Control | 10.91 ± 0.33 (15) | 10.56 ± 0.33 (14) | 0 | 6.66 |
| 17-hydroxyprogesterone | 11.19 ± 0.35 (15) | 10.83 ± 0.37 (15) | 0 | 0 |
| Methyl farnesoate | 11.21 ± 0.23 (15) | 11.19 ± 0.25 (13) | 0 | 13.33 |

Notes: Number of females is indicated between brackets. Percentages of molting and mortality are also indicated.

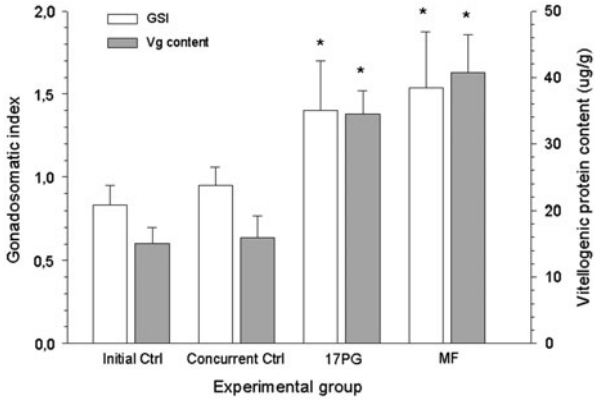


Figure 1. GSI and vitellogenic proteins (Vg) content in the ovary of *N. granulata* females at the end of the *in vivo* assay. Ctrl: Control; 17PG: 17-hydroxyprogesterone; MF: methyl farnesoate. Asterisks indicate significant ($p < 0.05$) differences with respect to concurrent control.

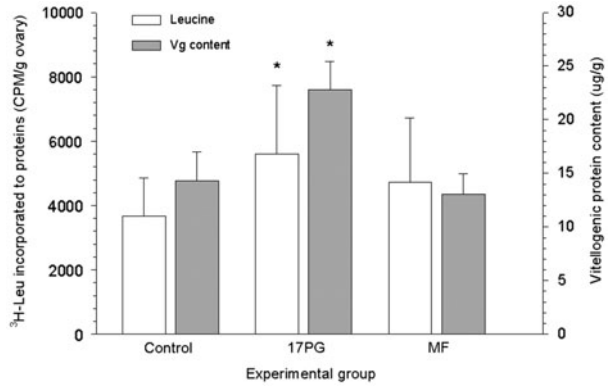


Figure 2. Levels of tritiated leucine ($^3$H-Leu) incorporated with ovarian proteins and vitellogenic proteins (Vg) content in ovarian pieces of *N. granulata*, incubated *in vitro* with 15 µM of 17-hydroxyprogesterone (17PG) or MF. Asterisks indicate significant ($p < 0.05$) differences with respect to control.

No significant differences ($p > 0.05$) in both the HSI (an overall mean of $4.30 \pm 0.16$, $N = 42$) and the hepatopancreatic Vg protein content (an overall mean of $54.52 \pm 6.02$ µg/g, $N = 42$) were seen among all treatments. Table 2 shows mean titers of hemolymphatic vitellogenin, detected at the beginning, middle, and end of the assay; females treated with either MF or 17PG showed significantly higher levels ($p < 0.05$) than control at the end of the assay.

### In vitro *assay*

Results from the first *in vitro* assay showed that the level of tritiated leucine incorporated to proteins in ovaries treated with 17PG was significantly higher ($p < 0.05$) than that of controls (Figure 2). Similarly, results from the *in vitro* hormonal treatment assay showed a significantly higher ($p < 0.05$) content of Vg proteins in ovaries incubated with 17PG with respect to control (Figure 2). No significant effects ($p > 0.05$) of MF were seen on protein synthesis or Vg protein concentration.

## Discussion

No differences in survival or molting were observed among treatments during the *in vivo* assay. In several crustacean species, the post-reproductive period represents a stage of reproductive quiescence, during which animals become highly refractory to environmental stimuli such as temperature and photoperiod (Waddy & Aiken 2000). During this period, females of *N. granulata* mainly allocate the available energy to somatic growth, leaving the ovary insensitive to stimulation by environmental changes. Moreover, eyestalk ablation during the post-reproductive period only triggered molting, likely by suppressing the source of the molt-inhibiting hormone (Stella et al. 2000). However, the administration of spiperone, a dopaminergic antagonist, was able to induce ovarian growth during the post-reproductive period of *N. granulata* (Zapata et al. 2003), presumably by stimulating the secretion of the gonad-stimulating hormone secreted by the thoracic ganglion (Cahansky et al. 2011).

Several *in vivo* assays provided evidence for the role of 17PG in promoting ovarian growth. For example, shrimp injected with 17PG showed faster ovarian growth (Yano 1987). In the crayfish *P. clarkii*, both 17PG and 17β-estradiol administered at the end of the pre-reproductive period increased both GSI and oocyte diameter (Rodríguez et al. 2002a). Similar effects have been reported in the crab *O. senex senex* by Reddy et al. (2006) together with an increase in the circulating

Table 2. Concentration (ng/µL) of vitellogenin in hemolymph at the start, middle (2nd week) and end (4th week) of the *in vivo* assay.

| | Vitellogenin hemolymphatic level (ng/uL) | | |
|---|---|---|---|
| Experimental group | Initial | Middle | End |
| Control | 252.60 ± 7.52 (15) | 176.36 ± 11.14 (15) | 156.32 ± 19.03 (14) |
| 17-hydroxyprogesterone | 249.78 ± 11.78 (15) | 172.78 ± 10.07 (15) | 241.39 ± 17.05 (15)* |
| Methyl farnesoate | 223.54 ± 17.24 (15) | 149.39 ± 9.75 (14) | 273.13 ± 14.19 (13)* |

Notes: Number of females is indicated between brackets. Asterisks indicate significant differences ($p < 0.05$) with respect to control.

vitellogenin level. In adult females of *N. granulata*, either injecting or feeding 17PG increased GSI in the three periods of the reproductive cycle (Zapata et al. 2003). In this study, feeding 17PG during the post-reproductive period produced a significant increase in both GSI and ovarian vitellins.

Several previous *in vitro* studies have reported stimulating effects of 17PG on ovarian growth. For instance, Tsukimura and Kamemoto (1991) observed a significant increase in oocyte diameter in ovaries of the shrimp *Penaeus vannamei* exposed to 17PG *in vitro*. Additionally, during the reproductive period of *N. granulata,* 17PG stimulated ovarian growth *in vitro* through incorporation of tritiated leucine into proteins (Zapata et al. 2003). Results from this study extend these observations and demonstrate a stimulating effect of 17PG on ovaries during the post-reproductive period, not only in terms of protein synthesis (incorporation of leucine to ovarian proteins), but also by showing that these are mainly vitellogenic proteins. Thus, this study demonstrates that both end points, incorporation of tritiated leucine into proteins and the concentration of vitellogenic proteins are sensitive variables that can be used to estimate ovarian growth.

Incorporation of MF to pellet food was shown in this experiment to be effective for stimulating ovarian growth; that is, at the end of the 4-week *in vivo* assay, a significant increase in both GSI and ovarian Vg protein content were observed. The *in vivo* effect of MF on crustacean reproduction has been studied in several decapod species (Laufer et al. 1992, 1998; Reddy & Ramamurthi 1998; Rodríguez et al. 2002a; Nagaraju et al. 2003; Nagaraju 2007; Balusbramanian et al. 2010; Suneetha et al. 2010). Particularly, pellets enriched with MF administered to the shrimp *P. vannamei* produced an increased fecundity and spawning (Laufer et al. 1992). A similar enriched diet for the crayfish *P. clarkii* produced a significant increase in both GSI and oocyte diameter (Laufer et al. 1998). In the same species, a significant increase in oocyte size was observed after injection with MF (Rodríguez et al. 2002a). However, neither shrimp nor crayfish show a distinct seasonal reproductive pattern as that reported for the crab *N. granulata*, whose post-reproductive period is clearly shifted to somatic growth (Stella et al. 2000; Zapata et al. 2003).

However, the stimulating effect of MF observed *in vivo* was not observed *in vitro*. A stimulating *in vitro* effect of MF has been reported on ovaries of the crayfish species *P. clarkii* (Rodríguez et al. 2002a) and *C. quadricarinatus* (Medesani et al. 2012), during different periods of the year. One possible explanation for the absence of a direct *in vitro* effect of MF on the ovary of *N. granulata*, observed during the post-reproductive period together with the stimulating effect observed *in vivo*, is that MF exerts its effect indirectly. It may be that the stimulating effect is exerted during this period by hormones other than MF, but whose secretion could be stimulated by MF. Some steroids of gonadal origin (such as 17PG), as well as the gonad-stimulating hormone (GSH) secreted by brain and thoracic ganglion (Fingerman 1997), would be plausible candidates. Moreover, determination of 17PG levels after MF *in vivo* administration could be a suitable methodology to test this hypothesis. In the studied species, both 17PG and spiperone (a dopaminergic antagonist) were able to stimulate ovarian growth during the post-reproductive period (Zapata et al. 2003). Spiperone would trigger the secretion of GSH, since an increased protein synthesis was seen in the ovary of the studied species during the post-reproductive period, but only when the ovary was incubated with thoracic ganglion (Cahansky et al. 2011).

No differences in HSI or Vg protein content were observed in the hepatopancreas among treatments. However, Vg hemolymphatic level at the end of the *in vivo* assay was higher in females treated with either 17PG or MF compared to control. These results, taken together, could be explained as a balance between production and exportation of vitellogenin by the hepatopancreas. If both processes were augmented at the same time, it would be reasonable to expect no changes in the hepatopancreatic Vg protein level, but an increase in hemolymph Vg level. This could explain the results obtained for the hormonally treated crabs. Concerning controls, the decreased hemolymph Vg level observed over the experimental period suggests a progressively less exportation of Vg from the hepatopancreas, a result expected in normal animals of the studied species during the post-reproductive period (López Greco & Rodríguez 1999).

The results of this study show that *in vivo* administration of either 17PG or MF through diet could be used as an efficient way to induce ovarian growth during the post-reproductive period without affecting survival or molting, especially in decapods species with a markedly seasonal reproductive cycle such as *N. granulata*. The use of hormonally enriched pellets is a non-invasive and practical option for aquaculture purposes compared to other methods of hormone administration.

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