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Preliminary evaluation of the effect of juvenile hormone III and methyl farnesoate on spermatophore quality of the white shrimp, *Litopenaeus vannamei* Boone, 1931 (Decapoda: Penaeidae)

Evaluación preliminar del efecto de la hormona juvenil III y del metil farnesoato en la calidad de espermatóforos del camarón blanco, *Litopenaeus vannamei* Boone, 1931 (Decapoda: Penaeidae)

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Resumen.- Este estudio preliminar es un primer paso en la exploración del efecto de la hormona juvenil III (HJ III) y el metil farnesoato (MF) sobre la calidad de espermatóforos de *Litopenaeus vannamei*. La HJ III aplicada cinco veces a una dosis de 109 ng g^{-1} de peso corporal (p.c.) durante 58 días, no mejoró el conteo de espermatozoides en comparación con el grupo control (P>0,05). La anormalidad de espermatozoides disminuyó en las regeneraciones sucesivas de espermatóforos de manera similar en machos tratados con HJ y machos control (P>0,05). Por el contrario, un total de cinco aplicaciones semanales de MF a 120 ng g^{-1} p.c. causó un incremento significativo en número de espermatozoides y una disminución en anormalidad de espermatozoides (P<0,05). Una dosis mayor de MF (1200 ng g^{-1} p.c.) no causó un efecto significativo sobre la calidad de los espermatóforos (P>0,05).

Palabras clave: Camarones penaeoideos, Dendrobranchiata, hormona juvenil, metil farnesoato, reproducción

Introduction

Reproduction facilities based on pond-grown animals require males that generate acceptable levels of high quality sperm. Males from earthen ponds, regardless of size, show low sperm levels and high rates of abnormal sperm (Leung-Trujillo & Lawrence 1985, Alfaro & Lozano 1993, Alfaro 2001, Ceballos-Vásquez *et al*. 2003).

Juvenile hormones (JH 0, I, II, III) play a major role in controlling larval development, stimulating vitellogenin synthesis, and controlling female reproductive development in Insecta (Downer & Laufer 1983). As in insects, methyl farnesoate (MF, unepoxidated precursor of JH III), synthesized and secreted by the mandibular organ in crustaceans, seems to stimulate reproduction in both males and females acting as a gonadotropin and also a morphogen (Laufer *et al*. 1988, Homola *et al*. 1991, Rotllant *et al*. 2000, Laufer & Biggers 2001).

The function of juvenile hormone-like compounds in crustaceans has been subject to debate (Wilder *et al*. 1995, Homola & Chang 1997, Abdu *et al*. 2001); in *Procambarus clarkii* Girard 1852 and *Libinia emarginata* Leach 1815, MF elevations stimulate ovarian maturation (Laufer *et al*. 1998, Que-Tae *et al*. 1999); however, in *Cherax quadricarinatus* Martens, 1868 there is no effect on female reproduction (Abdu *et al*. 2001). In *L. emarginata*, there are different male morphotypes and their reproductive behavior and the reproductive system development are associated with different levels of MF in the hemolymph (10.7 to 67.2 ng mL^{-1}; Homola *et al*. 1991, Sagi *et al*. 1993). In *Penaeus japonicus* Bate 1888, JH and MF were not detected in males, immature females, nor maturing females (Wilder & Aida 1995). However, MF has been identified in other penaeoid shrimps, including *Litopenaeus vannamei* Boone, 1931 and *L. stylirostris* Stimpson, 1874 (Laufer & Biggers 2001). In *L. vannamei,* JH III and MF significantly increased the diameter of late perinucleolus stage oocytes from isolated immature ovaries (Tsukimura & Kamemoto 1991). Fecundity in *L. vannamei* was increased by MF, probably as an indirect result of Y-organ stimulation to synthesize and secrete ecdysteroids, which accumulate in the ovaries (Laufer 1992, Laufer *et al*. 1997).

No study has been published on the role that juvenoids may play on spermatophore quality of penaeoid shrimps; therefore, this is a preliminary study designed to explore the possible effect of JH III and MF injection on spermatophore quality of *L. vannamei*.

Material and methods

JH III experiment

Ten young males (b.w. = 23.0 ± 2.8 g) were stocked in an 18 m^2 maturation tank at Estación de Biología Marina, Puntarenas, Costa Rica. Water exchange was kept at 20% per day; salinity was 32 ppt and temperature was maintained at 28°C. Photoperiod was natural (13 h light: 11 h dark), with reduced light intensity (10 – 43 lux). Animals were fed at 1.6% b.w. of a dry commercial feed plus fresh frozen sardine and squid at 4% b.w., and acclimatized for one week. Males were randomly assigned to two treatments: a) JH III injection (n = 5), and b) control group (n = 5).

JH III was prepared by dissolving the hormone (Sigma Co.) in ethanol to 2.5 mg mL^{-1}. A working solution was then prepared by diluting 0.10 mL of the concentrated stock to 10 mL of sterile saline solution (0.85% NaCl), and kept under refrigeration during the experimental period. Males were injected laterally in the second abdominal segment with 0.10 mL (2 500 ng of JH III) to give a dose of 109 ng g^{-1} b.w., at days 1, 9, 30, 43, and 58. Control males received 0.10 mL of the vehicle solution (0.10 mL ethanol to 10 mL saline solution), administered the same days.

Spermatophores were manually ejaculated at days 8, 29, 42, 57, and 70, and analyzed for sperm count, and percentage of abnormal sperm, as previously described (Alfaro & Lozano 1993). Measurements were repeated three times for each male.

MF experiment

Twenty males (b.w. = 27.5 ± 2.1 g) were stocked in an 18-m^2 maturation tank. Culture conditions were similar to the first experiment, and animals were fed at 15% b.w. with fresh frozen sardine and squid (1:1). Two males were used for baseline spermatophore condition at initiation, and the other males were randomly assigned to three treatments: a) control group (n = 6), b) MF injection at 120 ng g^{-1} b.w. (n = 6), and c) MF injection at 1200 ng g^{-1} b.w. (n = 6).

MF was prepared by dissolving the hormone (Echelon Biosciences Inc.) in ethanol to 10 mg mL^{-1}. MF at 120 ng g^{-1} was then prepared by diluting 0.07 mL of the concentrated stock to 10 mL of ethanol, and injecting 0.05 mL. MF at 1200 ng g^{-1} was prepared by diluting 0.7 mL of the stock solution to 10 mL ethanol, and injecting 0.05 mL. Solutions were kept at –10ºC during the experimental period. Males were injected laterally in the second abdominal segment at days 1, 8, 15, 22, and 29. Control males received 0.05 mL of the vehicle solution (ethanol), administered the same days. Analysis of spermatophores was performed on day 36 as indicated in the previous experiment; successive ejaculations were not performed to exclude this quality enhancing factor as it was observed in the JH experiment.

Statistical Analyses

Data on sperm count and sperm abnormalities were transformed using squared root of Y + 3/8 and arcsine of squared root of Y, respectively, to make the variance independent of the mean (Ott 1984). Transformed data from the JH experiment were analyzed with 2-sample t test at $\alpha = 0.05$ (Ott 1984), comparing mean values between treatments at each successive ejaculation. Complementary, mean values within treatments were compared at first and last ejaculation using paired t test at $\alpha = 0.05$ (Ott 1984). Transformed sperm counts and abnormalities from the MF experiment were analyzed with one way analysis of variance and Tukey´s w procedure at $\alpha = 0.05$ (Ott 1984). Statistical analyses were performed with Minitab program. Figures present untransformed data as mean values ± standard deviation.

Results

Mortality was very low for the JH experiment; being 1/5 and 0/5 for JH and control group, respectively. The control group (Fig. 1) showed an increase in sperm count in successive regenerations, reaching an average of 18 and 17 million sperm per compound spermatophore at ejaculation 4 (day 57) and 5 (day 70), respectively. Sperm count at day 70 was statistically higher than at day 8 ($P<0.05$). The standard deviation lines, for the control group, indicate an increased variability between replicates as the study progresses. On the other hand, JH III-injected males at 109 ng g^{-1} b.w. experienced a low or no sperm improvement in successive regenerations; no statistical differences were detected between day 8 and 70 ($P>0.05$). The high variability measured for the control group did not allow detecting significant differences between treatments ($P>0.05$).

In successive spermatophore regenerations, sperm abnormalities (Fig. 1) decreased at a similar rate between treatments. Control males with abnormalities around 60% at first ejaculation, decreased to 46% at fifth regeneration, and JH treated males reduced abnormalities from 80% (ejaculation 1) to 50% (ejaculation 5). No statistical differences were detected between treatments at each ejaculation ($P>0.05$).

MF was tested at two doses, with five injections being applied weekly (Fig. 2). Mortality was 3/6 for control and MF 1 200 treatments, and 2/6 for MF 120. This

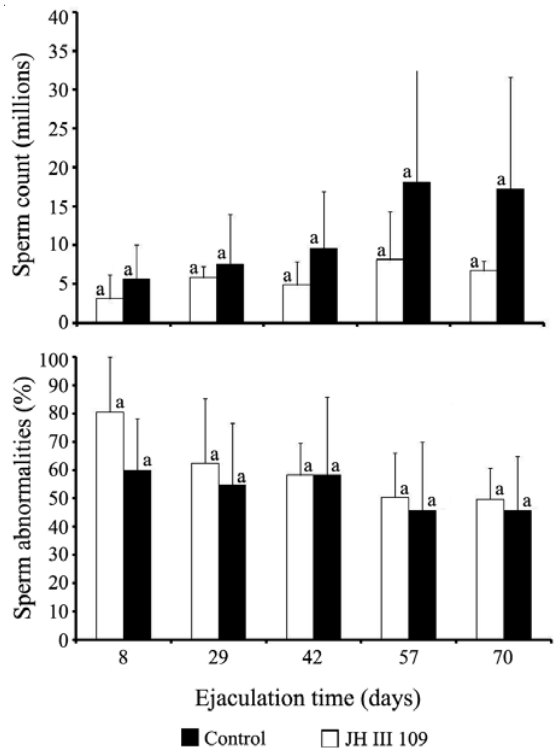

Figure 1

Evaluation of sperm counts and sperm abnormalities after successive spermatophore ejaculations for *Litopenaeus vannamei* injected with five doses of JH III (109 ng g^{-1} b.w.) or vehicle solution (control group). Bars with same letters within each time sample indicate no statistically significant differences between JH-treated and control males (P>0.05)

Evaluación del conteo de espermatozoides y anormalidad de espermatozoides en regeneraciones sucesivas de espermatóforos de *Litopenaeus vannamei* inyectados con cinco dosis de HJ III (109 ng g^{-1} p.c.) o solución vehículo (grupo control). Barras con las mismas letras dentro de cada tiempo de muestreo indican diferencias estadísticas no significativas entre machos tratados con HJ y controles (P>0,05)

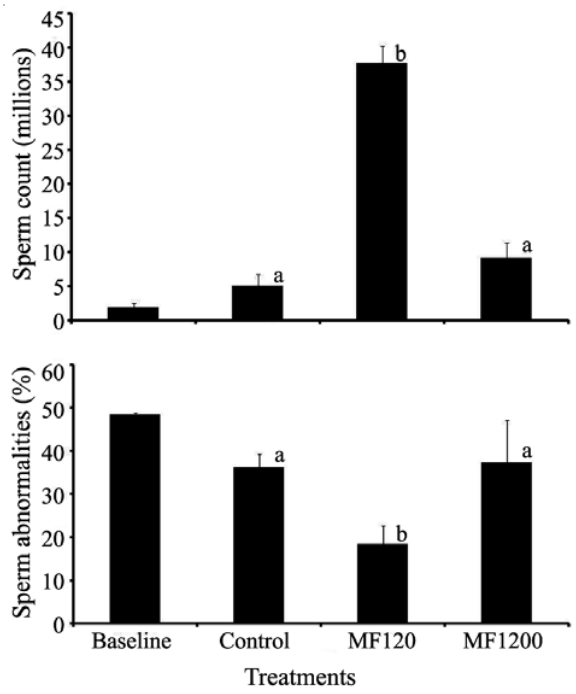

Figure 2

Evaluation of sperm counts and sperm abnormalities after a single ejaculation for *Litopenaeus vannamei* injected with five weekly doses of MF (120 or 1200 ng g^{-1} b.w.) or vehicle solution (control group). Baseline values were included as a reference. Bars with different letters indicate statistically significant differences between treatments (P<0.05)

Evaluación del conteo de espermatozoides y anormalidad de espermatozoides luego de una sola eyaculación de *Litopenaeus vannamei* inyectados con cinco dosis semanales de MF (120 o 1200 ng g^{-1} p.c.) o solución vehículo (grupo control). Valores base fueron incluidos como referencia. Barras con letras diferentes indican diferencias estadísticas significativas entre tratamientos (P<0,05)

mortality could be induced by the amount of ethanol injected (50 µL), which was quite high, compared to a previous study (2 µL; Alfaro 1996) and the JH experiment (1 µL). The response in sperm counts indicates a significant improvement (P<0.05) for this variable at 120 ng g^{-1} MF. The response measured at 1200 ng g^{-1} was not different (P>0.05) from the control group. Sperm abnormalities (Fig. 2) also indicate a positive effect of 120 ng g^{-1} MF on this quality indicator (P<0.05). No statistical differences (P>0.05) were detected between control group and MF at 1200 ng g^{-1}.

Discussion

Artificial ejaculation under controlled reproduction conditions induced a positive effect on the quality of *L. vannamei* spermatophores as indicated by the average increase in sperm count (P<0.05) and the average reduction in sperm abnormalities measured for the control group of the JH experiment. These findings agree with previous reports dealing with the positive influence of culture conditions (Alfaro & Lozano 1993) and age on sperm quality (Ceballos-Vásquez *et al*. 2003).

The dilution of JH in saline solution was not an adequate protocol based on the hydrophobic nature of juvenoids; however, Fig. 1 shows a clear difference in response between control group and JH III group, suggesting that JH injections at 109 ng g^{-1} caused some negative effect on sperm count after successive ejaculations. Complementary, no effect of JH III in combination with MF was detected on oocyte growth in *P. clarkii* (Rodríguez *et al*. 2002), but in the crab *Chasmagnathus granulata*, JH III (diluted in crustacean saline) increased the gonadosomatic index during the pre-reproductive period (Zapata *et al*. 2003). The injection of serotonin and JH III in *L. stylirostris* females did not induce ovarian maturation (Alfaro *et al*. 2003).

Contrary to JH III, MF at a similar dose (120 ng g^{-1}) apparently induced a positively significant response in sperm counts and sperm abnormalities ($P<0.05$). During the 36 days of treatment after five injections of MF at 120 ng g^{-1}, 27.5 g males improved their spermatophore quality in terms of an increase in sperm count and a decrease in sperm abnormality. However, MF at 1 200 ng g^{-1} (1.2 µg g^{-1}) did not improve significantly ($P>0.05$) those quality parameters. Variance among replicates was low and the effect of MF was so strong that a clear and significant difference was detected regardless of small sample size; however, this statement requires further experimental confirmation using more replicates. A similar pattern was observed in the branchiopod, *Triops longicaudatus* (Tsukimura *et al*. 2006); the authors reported that low doses of MF (0.75 – 3.8 µg g^{-1}) appeared to have an effect on fecundity, but higher doses (10 µg g^{-1}) reduced somatic growth.

A MF dose of 120 ng g^{-1} represents around 240 ng mL^{-1} hemolymph, based on the relation of 0.5 mL hemolymph per g b.w. (Shafir *et al*. 1992), or 3300 ng male $week^{-1}$. This dose is lower than the levels of MF used in *P. clarkii* to induce maturation (1000 - 2000 ng female day^{-1}; Laufer *et al*. 1998), and much higher than the levels measured for the reproductive male morphotype of *P. clarkii* (< 0.5 ng mL^{-1}) and the non-reproductive male type (1.3 ng mL^{-1}; Laufer *et al*. 2005). It is also similar to the doses of MF tested on *C. quadricarinatus* (4500 - 9000 ng animal $week^{-1}$) without any effect on reproduction (Abdu *et al*. 2001). In eyestalk ablated females of *L. emarginata* hemolymph levels of MF are 2 - 3.5 ng mL^{-1} (Que-Tae *et al*. 1999), and the level measured in large abraded males of this species is 67.2 ng mL^{-1} (Homola *et al*. 1991). These findings suggest that shrimp can not use JH III in place of MF, and provide preliminary evidence that MF is a reproductive hormone in penaeoid shrimps.

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