Effects of androgenic gland ablation on growth, sexual characters and spermatogenesis of the white shrimp, *Litopenaeus vannamei* (Decapoda: Penaeidae) males

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Abstract

This research was implemented to study the effects of androgenic gland ablation in the white shrimp *Litopenaeus vannamei* to explore sex reversal potential as an alternative technology for monosex female mariculture based on the sex dimorphic growth of this species. The surgical procedure was applied to male postlarvae (PL) at different ages, after external sex differentiation, as well as in sex undifferentiated PL. Andrectomized males regenerated normal appendices including pereopods and pleopods; however, body growth and relative size of regenerated petasmas and appendices masculinae were statistically inferior ($P < 0.05$) to control males. Spermatogenesis in andrectomized males was active, but a phenomenon of degradation of spermatids and reproductive tissues was detected. No sex reversal was accomplished regardless of PL age from sex undifferentiated stages (PL38) to sex differentiated stages (>PL55). The complete regeneration of sexual characters in andrectomized *L. vannamei* (Dendrobranchiata) is different from previous reports from Decapoda.

Keywords: Penaeidae, androgenic gland, *Litopenaeus vannamei*, sex reversal, shrimp

Introduction

Malacostracan crustaceans control sex differentiation by the androgenic gland (AG) that releases the androgenic hormone. This insulin-like peptide

has been identified in many crustaceans including amphipods, isopods and decapods (Ventura, Rosen & Sagi 2011). In decapods, the insulin-like androgenic hormone (IAG) was found to regulate the masculinization of primary and secondary sexual characters (Ventura & Sagi 2012).

In the family Penaeidae, the complete cDNA of IAGs, of the giant tiger prawn *Penaeus monodon*, the kuruma prawn *Marsupenaeus japonicus* and the Chinese shrimp *Fenneropenaeus chinensis* (Li, Li, Sun & Xiang 2012) have been reported. The definitive demonstration of the AG structure and location by molecular probes has been done in *P. monodon* and *F. chinensis* (Banzai, Ishizaka, Asahina, Suitoh, Izumi & Ohira 2011; Mareddy, Rosen, Thaggard, Manor, Kuballa, Aflalo, Sagi, Paterson & Elizur 2011; Li *et al.* 2012).

In the most economically important penaeid species: the white shrimp *Litopenaeus vannamei*, the complete sequence of the IAG gene was recently reported (Vázquez-Islas, Garza-Torres, Guerrero-Tortolero & Campos-Ramos 2014). The histology of the presumptive AG of *Litopenaeus*, including the southern shrimp *Litopenaeus setiferus*, the white shrimp *Litopenaeus occidentalis*, the blue shrimp *Litopenaeus stylirostris* and *L. vannamei* has been partially described (Campos-Ramos, Garza-Torres, Guerrero-Tortolero, Maeda-Martínez & Obregón-Barboza 2006; Garza-Torres, Campos-Ramos & Maeda-Martínez 2009; Alfaro-Montoya & Hernández 2012; Vázquez-Islas *et al.* 2014). These findings indicate that the AG in *Litopenaeus* is associated to the terminal ampoule and stretched

along the distal region of the descending vas deferens. The aim of this study is to evaluate the effects of the ablation of the presumptive AG (andrectomy) associated with the terminal ampoules of *L. vannamei* on growth, male sexual characters and spermatogenesis.

Materials and methods

Evaluation of *L. vannamei* dimorphic growth under pond and tank culture

The growth of 358 organisms cultured in a commercial semi-intensive earthen pond in Golfo de Nicoya, Costa Rica, was recorded monthly as body weight (BW) from postlarvae stage 12 (PL12) to PL108. Conventional aquaculture systems for *L. vannamei* harvest 3-month old animals; therefore, to evaluate dimorphic growth after harvesting age, a complementary tank culture experiment was implemented. Three groups of 160 animals each were harvested from commercial ponds at 21, 20 and 16 g BW for groups A: rainy season culture, B: rainy-dry season culture and C: dry-rainy season culture respectively. Each group was grown in a separated shaded external tank (18 m²; 20 MT; 9 shrimp per m²) at different periods of the year, allowing to compare performance under dry and rainy seasons. Culture time was different for each group depending on actual growth rate and it lasted until reaching at least 30 g for females at Estación de Biología Marina (EBM) in a flow-through system, using new water pretreated by high-pressure silica sand filtration and sedimentation. Animals were fed at 3% BW of a dry commercial feed (Nicovita[®], 30% protein, Callao, Perú) supplemented with fresh frozen sardines (*Opisthonema* sp.). Sex and BW was monitored monthly in the three groups by population samples of $n = 20$ animals.

Identification of the AG

Bilaterally eyestalk ablated *L. vannamei* males ($n = 3$, 30 g BW), were anaesthetized in cold water (20°C) 8 days post eyestalk ablation, and andrectomized by removing the coxas of their fifth pereopods and the associated terminal ampoules. Samples of ampoules were fixed in Davidson's solution for 24 h, dehydrated in ethyl alcohol, paraffin embedded and serially sectioned in cross-orientation. Sections were stained in Hematoxylin and Eosin.

Regeneration of pereopods and male sexual characters

Litopenaeus vannamei postlarvae were collected from commercial farms after 15 days of culture (PL 25–35), and transported to EBM for further culture in laboratory tanks (volume = 330 L) until reaching the following PL stages. Three groups of males (MA: $n = 25$, PL180, MB: $n = 74$, PL70 and MC: $n = 60$, PL55 to 107, See Table 1) were andrectomized and monitored for regeneration of pereopods, petasmas and appendices masculinae. Animals were mounted dorsally on Velcro straps or moulding clay under a dissecting stereo microscope. Petasma identification was done by removing the left first pleopod. Identified males were andrectomized. The coxas of the fifth walking legs were removed, as well as the terminal ampoules, the adjacent AGs, and vasa deferentia using fine tweezers. The appendix masculina associated with the left second pleopod was removed leaving the right pleopod intact as control. The regeneration of the ablated pleopod served as an additional tool to monitor the effects of AG ablation.

Andrectomy of sex differentiated individuals

Litopenaeus vannamei postlarvae from group B (previous experiment: PL70, BW = 4.36 ± 1.44 g) were further evaluated against a control group for body growth and re-development of sexual characters based on the following treatments:

Andrectomy (AN): As described above. First and second left pleopods were removed ($n = 34$).

Control (CO): removal of fifth walking legs damaging neither the coxa nor the ampoule-vas deferens. First and second left pleopods were also removed ($n = 28$).

Males from both treatments were maintained in the same tank (water volume = 330 L) under a flow-through system, using new water pretreated by high-pressure silica sand filtration and sedimentation. Salinity was 32 ppt, temperature was maintained at 28°C, and the photoperiod was 13:11 (L:D). Animals were fed daily *ad libitum* with Nicovita[®] feed (30% protein). After 4 months of culture, males from both treatments were inspected for the presence of ampoules/spermatophores (CO) or absence of ampoules/spermatophores (AN), and measured in terms of BW (g), total length (TL, mm; measured from

Table 1 Evaluation of microsurg-
eries of fifth pereopods on *Litopenaeus*
vannamei males

Parameter	Experimental groups		
	MA	MB	MC
Number of microsurgeries	25	74	60
Culture time (months)	8	4	3
Initial PL stage	180	70	55–107
Initial size (mm)	97.8 ± 6.4	69.1 ± 7.8	36.6 ± 3.6
Initial weight (g)	–	4.36 ± 1.44	0.50 ± 0.23
Surgery mortality (%)	24	16	23
Number of final survivors	14	47	12
Survival (%)	74	76	26
Final size (mm)	117.9 ± 7.7	110.5 ± 7.3	54.4 ± 12.3
Final weight (g)	22.46 ± 4.79	18.19 ± 3.52	–
Regeneration of fifth pereopods (%)	100	100	100
Regeneration of left petasma (%)	100	100	100
Regeneration of left appendix masculine (%)	–	100	100

the eyestalk base to the tip of telson), regenerated petasma length (PEL) and petasma width (PEW, mm) and regenerated appendix masculina length (APL) and appendix masculina width (APW, mm).

Ablation of sex undifferentiated postlarvae

Litopenaeus vannamei postlarvae at PL30 were collected from a commercial farm, transported to EBM for further culture in laboratory tanks. Sex undifferentiated PL38 (BW = 0.052 ± 0.015 g) were selected for the following treatments:

Ablation (ABL): removal of fifth walking legs at PL38 as described above ($n = 60$).

Control (CO): Non treated group of PL38 ($n = 92$).

Both groups were cultured in separated tanks (330 L), since gross external signs of andrectomization are not evident at this young stage. Culture conditions were as indicated previously and the experiment lasted until reaching clear sex differentiation after PL82, and measured in terms of BW and TL.

Spermatogenesis in andrectomized adult males

Andrectomy was performed on mature males (6 months old, $n = 4$). Males were dissected to remove testicular lobes for histological examination of spermatogenesis 12 months post andrectomy and were compared to histological sections from intact males. Tissues were fixed as described

previously, paraffin embedded, sectioned and stained in Hematoxylin and Eosin.

Statistics

Body weights and lengths were analysed using *t*-test for independent samples, at $P < 0.05$ (Ott 1984), using the program GNU PSPP version 0.8.1 (Boston, MA, USA). Male petasma and appendix masculina measurements were normalized as follows: relative petasma length, RPEL = PEL/TL; relative petasma width, RPEW = PEW/TL; relative appendix masculina length, RAPL = APL/TL; relative appendix masculina width, RAPW = APW/TL. Normalized data were analysed by linear regression and *t*-test for independent samples, at $P < 0.05$ (Ott 1984). The Levene statistic was applied to test for homogeneity of variance. Values are expressed as mean ± SD. The Chi-square test of independence was used to compare male proportions in sex undifferentiated postlarvae (Ott 1984).

Results

Dimorphic growth

Litopenaeus vannamei cultured in tanks after reaching market size (of around 20 g BW) exhibit a clear sex related dimorphic growth, in terms of BW ($P < 0.05$). The growth of young shrimp in commercial earthen ponds from PL 12 to PL 108 (BW = 12 g) did not show any

statistical difference between sexes ($P > 0.05$; Fig. 1). Figure 1 summarizes the growth pattern followed by males and females in three farm-harvested populations, cultured during different seasons. Depending on environmental conditions, each group of animals grew at different rates, with group A: rainy season reaching 30 g for females in 3 months, group B: rainy-dry season reached 30 g after 5 months, and group C: dry-rainy season reached 30 g after 10 months. On the other hand, males showed slower growth rates than females in each group, with statistically significant differences ($P < 0.05$).

Identification of *L. vannamei* AG

Serial sections of terminal ampoules (Fig. 2a), from bilaterally eyestalk ablated males, revealed the presence of a massive homogeneous group of

cells organized in grape-like clusters at the distal section of the terminal ampoules (AG in Fig. 2b–d).

Sex characteristics regeneration in males (PL55 and up)

The experimental groups (Table 1) responded similar to the surgical procedure, reaching between 16 and 24% mortality; however, final survival was inferior in the group of smallest males (C). Regardless of size, from 97.8, 69.1 and 36.6 mm TL, males from the three groups regenerated normal fifth pereopods similar to the intact pereopods; moreover, the first and second pleopods were also regenerated to apparently normal size as compared to unamputated pleopods, developing a normal petasma and appendix masculina. Development of gonopores was not studied in these experimental males.

Figure 1 Average weight of male and female *Litopenaeus vannamei* grown under pond and tank culture at different seasons. Different letters indicate statistically significant differences ($P < 0.05$).

Figure 2 The presumptive AG of *Litopenaeus vannamei*. (a) Terminal ampoules, (b) cross-section of terminal ampoules at distal region, scale bar = 200 μ m, (c) the AG mass, scale bar = 200 μ m, (d) AG cells, scale bar = 20 μ m. AG, androgenic gland; AGC, androgenic gland cells; TA, terminal ampoule; MSX, muscle layer; EPT, epithelial lining.

Figure 3 Control (a–c) and andrectomized (b–d) males showing regenerated fifth pereiopods and left petasma of similar size to un-amputated appendices. Andrectomized male does not present gonopores, terminal ampoules and spermatophores. SP, spermatophore; TA, terminal ampoule; PER5 REG, pereiopod fifth regenerated; NOR, normal; G, gonopore.

Andrectomy effects in sexually differentiated and undifferentiated individuals

Animals with fifth legs surgery at PL70 showed a complete regeneration of the petasma and the appendix masculina in control animals (Fig. 3a) as well as in andrectomized individuals (Fig. 3b). The only apparent difference between andrectomized and control individuals could be seen in the regeneration of the fifth pereiopods without gonopores and associated ampoules/spermatophores in the andrectomized (Fig. 3d) compared to control (Fig. 3c).

Andrectomized males after 4 months of culture showed significantly lower growth in weight (Fig. 4a) and length (Fig. 4b) than control males ($P < 0.05$). Complementarily, the dispersion pattern measured (Fig. 5a) separates the two groups of males based on their linear regression model and correlation coefficient. Andrectomized males presented a moderate positive correlation ($r = 0.57$) and the control group showed a weak negative correlation ($r = -0.21$). Andrectomized males developed significantly smaller petasmas and appendices masculinae, in terms of normalized length and width (Fig. 5b, $P < 0.05$). Regenerated pleopods developed into apparently normal size appendices as compared to unamputated appendices.

Spermatogenesis was observed in andrectomized adult males after 12 months of culture; testicular lobes show different cellular stages including spermatocytes and spermatids; the basic histology of testis from andrectomized males was compared

Figure 4 Body weight (a) and length (b) of andrectomized ($n = 26$) and control ($n = 21$) males, after 4 months of surgery at PL70. Treatments with different letters indicate statistically significant differences ($P < 0.05$).

against normal males cultured under the same experimental conditions. Histological alterations were evident in seminiferous tubules containing spermatids and in seminiferous ducts. The alterations that could be seen in the andrectomized animals can be described as follows: (1) seminiferous tubules with spermatids and degenerative masses

Figure 5 Scattergram and liner regression of body length and normalized petasma (PEL/TL) for andrectomized ($n = 26$) and control ($n = 21$) males (a), and relative growth of regenerated normalized petasmas in length and width (PEL/TL; PEW/TL) and appendices masculinae (APL/TL; APW/TL) from andrectomized and control males (b). Treatments with different letters indicate statistically significant differences ($P < 0.05$).

of spermatids, (2) seminiferous ducts with spermatids and degenerative nodules and (3) degenerative ducts with white spherical nodules (Fig. 6).

In addition to these alterations, spermatids in seminiferous tubules from andrectomized males were in early stage (Fig. 6b), without acrosome formation as observed in normal males (Fig. 6a). This study was not intended for comparing testicular activity between andrectomized and normal males; however, our control males always showed tubules with early and mid spermatids; mid spermatids are characterized by the presence of acrosome structures.

The sex undifferentiated postlarvae group (PL38, $n = 60$) had a surgery survival rate of 90%. The group developed petasmas in 23 PL82, and 24 PL82 developed female first pleopods, given a male ratio = 48.9%, statistically similar to the control group (56.5%; $P > 0.05$; Table 2).

Discussion

Sex dimorphic growth under culture conditions in *L. vannamei* was not observed under 12 g BW; however, after around 20 g the growth between

sexes diverges with time and it is dramatically and statistically superior for females after at least 3 months of culture, depending on the environment. Our findings are complementary to previous reports by evaluating the growth of sexes in adults (>30 g BW). The growth rate, in general, was better during the rainy season culture; the dry season is associated with low temperatures at night, reducing the growth of shrimp in commercial operations (Valverde-Moya & Alfaro-Montoya 2013). The final differences in weight, in the three experimental groups, were in the range of 5.0–8.6 g. A similar pattern was reported for *P. monodon* (Gopal, Gopikrishna, Krishna, Jahageerdar, Rye, Hayes, Paulpandi, Kiran, Pillai, Ravichandran, Ponniah & Kumar 2010); in this species the growth rate between sexes began to diverge in the range between 15 and 20 g BW. In *L. vannamei*, a previous study (Pérez-Rostro, Ramírez & Ibarra 1999) indicated that after 160 days of culture, the growth rate was significantly different between females (18.09 g) and males (16.96 g). Complementarily, Pérez-Rostro and Ibarra (2003) observed that the weight for growth divergence between sexes is affected by the environment, and

Figure 6 Histology of testicles from normal males (a) and andrectomized males (b–d) after 12 months of culture. (a) Normal seminiferous tubules (ST) with secondary spermatocytes (SPTC2) and mid spermatids (MSPMT), scale bar = 20 μ m, (b) seminiferous tubules with degenerated spermatids (DEGSPMT), scale bar = 20 μ m, (c) seminiferous duct (SD) with degenerated nodules (NOD) and vacuolated epithelium (EPT), scale bar = 20 μ m, (d) Testicular lobule with a macroscopic nodule, scale bar = 100 μ m. AC, acrosome, G, acrosomal granule.

Table 2 Fifth pereopods microsurgery of sex undifferentiated *Litopenaeus vannamei* postlarvae (PL38)

Parameter	Treatments	
	Surgery of fifth pereopods	Control
Initial PL stage	38	38
Number of replicates	47	92
Culture time (days)	45	90
Final PL stage	82	127
Female final size (mm)*	51.6 $\pm$ 5.6 ^a	55.4 $\pm$ 9.6 ^a
Male final size (mm)*	51.2 $\pm$ 6.8 ^a	55.7 $\pm$ 8.4 ^a
Female final weight (g)*	1.88 $\pm$ 0.49 ^a	2.37 $\pm$ 1.25 ^a
Male final weight (g)*	1.84 $\pm$ 0.60 ^a	2.36 $\pm$ 1.04 ^a
Number of males	23	52
Number of females	24	40
Male ratio (%)†	48.9 ^a	56.5 ^a

**t*-test for independent samples; sex mean values with the same letter, within treatment, indicate no statistically significant differences ($P > 0.05$).

†Chi-square: $\chi^2 = 0.7454$, *t* value (1) = 3.8415, $\alpha = 0.05$, n.s.

Moss and Moss (2006) reported that sexual dimorphism for growth in *L. vannamei* is not the result of feeding behaviour, suggesting a physiological advantage.

Concerning the cellular structure of the AG, the histology of the cells associated with the distal region of terminal ampoules from *L. vannamei*, resembles previous descriptions made from Penaeidae (Mareddy *et al.* 2011; Li *et al.* 2012); the mass of cells is organized in grape-like clusters, as described by Li and Xiang (1997) for *F. chinensis*. The AG cells described in this study are similar to the findings reported by Vázquez-Islas *et al.* (2014) from the subterminal region of the ampoules.

Andrectomized *L. vannamei* males regenerated normal fifth pereopods without gonopores and developed shorter and thinner petasmas and appendices masculinae in amputated pleopods, as compared to control males, during the 4 months of culture, following surgery. Amputated and normal appendices were apparently similar in size. A similar pattern in petasma growth was observed by Li and Xiang (1997) for *F. chinensis*, without statistical confirmation. In the Indian prawn *Fenneropenaeus indicus*, amputated petasmas and appendices masculinae did not regenerate in andrectomized adult males (Mohamed & Diwan 1991). In the giant freshwater prawn *Macrobrachium rosenbergii* males, andrectomy caused appendices masculinae

of unamputated second pleopods to grow slower than normal, and regenerated pleopods did not develop appendices masculinae at all (Nagamine, Knight, Maggenti & Paxman 1980). In the Australian red claw crayfish *Cherax quadricarinatus*, the fifth pereopods of andrectomized males did not regenerate (Tropea, Hermida & López-Greco 2011). Therefore, it seems that appendix and sexual characters regeneration in Pleocyemata and Dendrobranchiata is a complex phenomenon.

Growth of andrectomized males was lower than control males. Males from both treatments were cultured in the same tank, and the lower growth of andrectomized males is a novel observation in penaeid shrimps. This is similar to other decapod crustaceans in which the AG was suggested as a growth promoting agent as studied in AG implantation experiments in females (Manor, Aflalo, Segall, Weil, Azulay, Ventura & Sagi 2004) and AG ablation in males (Sagi, Cohen & Milner 1990). In *M. rosenbergii*, a reduction in growth following AG ablation was measured (Sagi *et al.* 1990), and silencing of the *Mr-IAG* gene by dsRNA causes the reduction in growth rate in males (Ventura, Manor, Aflalo, Weil, Raviv, Glazer & Sagi 2009), suggesting that IAGs can enhance growth in crustaceans. Contrary to the above, Li *et al.* (2012) suggested that in penaeids such as *P. monodon*, since females exhibit a higher growth rate, IAG is not necessary for enhancing growth because it only exists in male-specific AG (Mareddy *et al.* 2011). In *C. quadricarinatus*, AG implantation in females increased their growth rate and became similar to that of males (Manor *et al.* 2004); however, no growth effect was associated with AG ablation in males (Tropea *et al.* 2011). Our findings indicate that the AG of *L. vannamei* may have an enhancement effect on male somatic growth at early ages.

Spermatogenesis was altered in andrectomized males as indicated by histological evidence. In normal males, spermatogenesis advances in seminiferous tubules to mid spermatid stage; advanced spermatids and spermatozoa are only observed in vasa deferentia (Alfaro-Montoya 2013). These alterations are associated with the integrity of spermatids and the normal structure of seminiferous ducts, particularly with the integrity of the epithelial layer, generating reproductive tissue abnormalities that can reach macroscopic dimensions characterized by white spherical nodules (Fig. 6c,d).

The alterations observed in testicles of andrectomized males were associated with seminiferous tubules and ducts, containing spermatids at early stage. These alterations suggest that spermatid maturation is stopped and cellular degradation is activated, starting in seminiferous tubules, with the formation of degraded nodules that can reach macroscopical dimension affecting tubules and ducts. Such alterations could be the result of having an underdeveloped reproductive system, incapable of completing spermatogenesis at vas deferens nor assembling spermatophores in terminal ampoules (Alfaro-Montoya 2010). Andrectomy could also affect the normal process of spermatogenesis, interrupting spermatid maturation, as suggested for other crustaceans, including *M. rosenbergii* (Nagamine *et al.* 1980) and *F. indicus* (Mohamed & Diwan 1991). The degradation of seminiferous tubules and ducts may indicate that the AG might also play a role in maintaining the reproductive system throughout the animal's life, as suggested by Tropea *et al.* (2011) for *C. quadricarinatus*.

Andrectomy of sexually undifferentiated individuals (PL38) did not induce any measurable shift in sex ratio, suggesting that sex reversal in *L. vannamei* requires AG ablation or molecular intervention in its specific hormone/s at younger stages than PL38, or that the removal of the AG via this method was incomplete. According to Garza-Torres *et al.* (2009) external sex differentiation occurs around PL32; however, the genital organs differentiate sooner (PL16). A complementary study by Campos-Ramos *et al.* (2006) indicates that external differentiation in *L. vannamei* begins around PL50, independently of size between 0.2 and 0.6 g.

This contribution demonstrated, for the first time, in an open thelyca shrimp: *L. vannamei*, that andrectomy causes a complete regeneration of appendices, including pereopods and pleopods. Andrectomized males showed a lower growth rate and smaller petasmas and appendices masculinae as compared to control males. Spermatogenesis in andrectomized males is active with degradation of spermatids and reproductive tissues. No sex reversal was achieved regardless of postlarvae stage starting at PL38.

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