



# Implant recognition and gender expression following ampoule-androgenic gland implantation in *Litopenaeus vannamei* females (Penaeidae)

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## ARTICLE INFO

### Article history:

Received 23 June 2016

Received in revised form 24 October 2016

Accepted 5 November 2016

Available online 11 November 2016

### Keywords:

Androgenic gland

Immune recognition

*Litopenaeus vannamei*

Penaeidae

Shrimp

Tissue implants

## ABSTRACT

Indirect evidence indicates that penaeids may have a W/Z sex heritability mechanism with the androgenic gland (AG) mediating masculine differentiation. The present study evaluated the immune response against grafts and the expression of primary and secondary male sexual characters in *Litopenaeus vannamei* females implanted with terminal ampoules and associated AGs. Experiments included grafting females at PL<sub>34–48</sub>. Grafting at PL<sub>37</sub> generated few cases of melanization exhibiting dark coloration covering parts or the whole implant while most implants showed white coloration, without any trace of melanization. Histology of melanized implants showed necrotic tissue, encapsulated by black depositions. These melanized capsules were externally surrounded by normal haemocytes and flat haemocytes in the middle. An experiment evaluating possible absorption of the grafts based on recovery, gross coloration, size of implants, and histology showed that Complete terminal ampoule-AG implants, Control-vas deferens implants and Abdominal muscle implants, recovered at day 7 and 15, exhibited gradual and statistically significant decrease in size, which was more evident for muscle implants. Grafts with normal appearance from this experiment remained viable during 7 and 15 days post-implantation; these tissues showed a normal cellular structure with neither signs of melanization nor haemocyte infiltration. *Appendices masculinae* did not develop in any implanted females or controls. Concerning petasma development, two females implanted with AG showed a male phenotype in their first endopodites, characterized by a straight shape at the distal region, a middle protuberance and absence of setae along the middle curved edge of the structure. Regenerated and intact endopodites of Control-proximal terminal ampoule females showed the typical female phenotype with a slender shape with a curved distal edge, without a middle protuberance, and with setae along the middle curved edge of endopodites. Implanted females showed no evidence of masculinization or abnormal development of oocytes. In the light of the present study the effects of AG transplantation on sex in penaeids should be further evaluated in younger PL stages.

**Statement of relevance:** This contribution presents new information on sex reversal technology on *Litopenaeus vannamei* based on androgenic gland implants. The findings are novel for the family Penaeidae concerning the immunological response to tissue grafting and the plasticity for sex reversal in this world aquaculture species.

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## 1. Introduction

The androgenic gland (AG) was first described in the crab, *Callinectes sapidus*, as an accessory gland without known function (Cronin, 1947). A few years later, Charmiaux-Cotton (1954) established its role in the regulation of male sexual characters. The AG releases an androgenic

hormone, that belongs to the insulin superfamily of peptides (termed Insulin-like androgenic gland hormone (IAG)), responsible for male sexual differentiation in malacostracan crustaceans (Ventura et al., 2011b).

Sex reversal in crustaceans has been accomplished through AG manipulation, including surgical removal or implantation of AG and IAG RNA interference (Ventura et al., 2011a, 2011b). In the fresh water prawn, *Macrobrachium rosenbergii*, sex reversal technology has reached a commercial level where gene silencing by RNA interference allows the production of neo-females (Ventura et al., 2011a) as a broodstock source for all-male postlarvae. Previous studies on *M. rosenbergii*

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demonstrated sex reversal by AG implantation or removal. Implantation of AG generated 90% of females with *appendix masculina* and 2/13 of the females showed spermatogenesis activity (Nagamine et al., 1980b).

Neither development of *appendix masculina* nor mature quelipedes were detected following AG ablation in *M. rosenbergii* while the testes and vasa deferentia were atrophied; complete feminization was achievable in males that were treated at early stages of development (Nagamine et al., 1980a). Alfaro et al. (2006) reported 17.98% of andrectomized males developed ovaries and female mating behavior, 2% laid eggs, and 1.28% were able to complete the larvication cycle and produced 100% all-male progeny. Achieving complete sex reversal depends on the stage of development of the manipulated individual (Ventura et al., 2011a).

In penaeid shrimps, sex reversal has not been accomplished by any means yet (Alfaro-Montoya et al., 2015). However, higher growth rates for females have been demonstrated in *Penaeus monodon* and *Litopenaeus vannamei* (Gopal et al., 2010; Alfaro-Montoya et al., 2015), suggesting that monosex shrimp culture is a biotechnological alternative for the industry (Ventura and Sagi, 2012). Moreover, the mechanism of sex determination has not been confirmed, but some evidence, like sex-linked markers on the maternal map, indicate that penaeid shrimps may have a mechanism similar to *M. rosenbergii*, with females being heterogametic (ZW; Benzie et al., 2001; Staelens et al., 2008; Zhang et al., 2007; Robinson et al., 2014). The location and morphology of the presumptive AG in *L. vannamei* were recently reported (Vázquez-Islas et al., 2014; Alfaro-Montoya et al., 2015) and the IAG gene was sequenced (Vázquez-Islas et al., 2014); however, there is no confirmation of actual IAG release from identified AG by means of Immunocytochemistry.

This study was designed to evaluate the immune response against implants of terminal ampoules and associated AGs in *L. vannamei*. In addition, the expression of primary and secondary male sexual characters in implanted females was studied.

## 2. Materials and methods

### 2.1. Experimental animals

*L. vannamei* postlarvae males and females 12 days after metamorphosis (PL<sub>12</sub>) were collected from commercial farms in Colorado de Abangares, Golfo de Nicoya, Costa Rica. Animals were transported to the Reproductive Physiology Laboratory at Estación de Biología Marina, Lic. Juan Bertoglia Richards (EBM), in Puntarenas. Tanks of 4 M.T. with flow-through water system were used; salinity was 32 ppt, temperature was maintained at 28 °C, and the photoperiod was 13:11 (L:D). Animals were fed daily at 3% of body weight (B.W.) with Nicovita® feed (30% protein).

### 2.2. Surgical procedure

Donor males were kept alive until dissection, and both ampoules and associated vas deferens were aseptically removed by pooling with fine tweezers the fifth pereopods from the coxa, expelling spermatophores from ampoules, and maintaining tissues in chilled (12–14 °C), sterile crustacean physiological solution (C.P.S.; Ro et al., 1990) for 11–21 min until transplantation into recipient females as previously described by Nagamine et al. (1980b), and Alfaro et al. (2009).

Females PL<sub>34–48</sub> were evaluated in two experiments: Experiment A implanted tissues removed from 30 subadult males (B.W. = 24.56 ± 3.58 g, T.L. = 116.94 ± 6.22 mm). Two different tissues were evaluated as treatments: distal terminal ampoule and associated AG located near the gonopore (DTA-AG) and proximal terminal ampoule without AG (Control PTA). Another experiment implanted tissues removed from 55 young males (B.W. = 9.32 ± 1.86 g; T.L. = 88.18 ± 5.33 mm), evaluating two different tissues in three treatments: complete terminal

ampoule with attached AG (CTA-AG), vas deferens (Control VD) and an intact control treatment (Control I).

Recipient postlarval females were immobilized on a dry molding clay base under a dissecting microscope. A small hole was made with a dissecting needle in the soft epidermis at the dorsal junction between cephalothorax and abdomen, then the tissue was taken with fine tweezers and implanted into the cavity located at the left of the heart (Alfaro et al., 2009). Equipment disinfection with ethanol 75% was performed before and after each surgical procedure.

### 2.3. Experimental design

Tissue implantation recognition and gender effect were evaluated in two experiments. Both experiments were treated as follows:

Experiment A: 98 females (Age = PL<sub>34–48</sub>; B.W. = 2.79 ± 0.38 g; T.L. = 58.09 ± 2.58 mm) distributed in 2 treatments: DTA-AG: n = 47, Control PTA: n = 51.

Experiment B: 109 females (Age = PL<sub>37</sub>; B.W. = 0.94 ± 0.37 g; T.L. = 39.82 ± 4.76 mm) distributed in 3 treatments: CTA-AG: n = 43, Control VD: n = 34, and Control I: n = 32.

A complementary assay (Experiment C) was undertaken to further evaluate the hypothesis proposed by Alfaro et al. (2009), concerning absorption mechanism for graft elimination. The condition of implants was studied based on recovery, gross coloration, size of implants and histology. This experiment included 104 animals (43 females and 61 males) distributed randomly in 3 treatments: CTA-AG: n = 35, VD: 39, abdominal muscle (M): n = 30. Both sexes were used as replicates since tissue recognition and haemocytic response are not affected by sex, but by the phylogenetic closeness between donor and recipient species (Lackie, 1986).

### 2.4. Tissue recognition analysis

Implants from Experiments A and B were analyzed in vivo seven days after surgery by classifying them into two gross categories, based on the general appearance of implants under a dissecting microscope: a) melanized implants, tissues with brown-black coloration, b) unmelanized implants, tissues with normal coloration.

Implants from Experiment C were removed from recipients at the end of the incubation period (7 and 15 days), catalogued based on three gross categories: a) undetected tissue, b) unmelanized, and c) melanized. Unmelanized and melanized tissues were measured before and after surgery. At the end of the experiment the implanted tissues were removed and fixed in Davidson's solution for 24 h and transferred to ethanol 50% for storage (Bell and Lightner, 1988). Samples were dehydrated in increasing ethanol concentrations, cleared and embedded in paraffin. Sections (8 µm) stained in hematoxylin and eosin at the Laboratorio de Patología Andrómeda, S.A., Guadalupe, San José, Costa Rica. Concurrently, an anatomical comparison of terminal ampoules-AG from normal and 15 days bilateral eyestalk ablated males was performed. Cellular structure and integrity of transplants were compared to non-implanted tissues by histological analysis as applied by Nagamine et al. (1980b) and Alfaro et al. (2009). Tissue structure descriptions were based on Bell and Lightner (1988).

### 2.5. Primary and secondary sex characters expression

Sex character expression was monitored by removing the first and second left pleopods with fine tweezers and observing the development of petasma and *appendix masculina*. Development of male genital papilla, body growth, normalized endopodite length of first pleopods as an index for petasma formation, and gonad histology were also analyzed in experimental females.

## 2.6. Statistical analysis

Endopodite length of first pleopods was normalized in relation to total body length; Shapiro-Wilk and Levene tests were performed in order to evaluate normality and homogeneity of the variance. Total weight, total length, and normalized endopodite length were statistically compared between treatments using T-student (Experiment A) and ANOVA (Experiment B). Additionally, a Student *t*-test was performed to compare implant size between day 1 and 15 (Experiment C). Statistical analyses were performed with R free license software (R Core Team, 2014).

## 3. Results

### 3.1. State of implants

In Experiments A and B, at 1 h, 24 h and 7 days post implantation, survival rates were higher for females with control implants. In both experiments, after 7 days of implantation, the survival rate was slightly higher for control females (Control PTA = 76% and Control VD = 74%) than AG implanted females (DTA-AG = 64% and CTA-AG = 56%). At the end of the experiment, DTA-AG had the highest mortality, while the other implanted groups showed relatively similar survival rates. Final survivals of AG implanted females were 32 and 49%, for experiment A and B, respectively; whereas Control I females at the end of the experiment reached 60% survival (Table 1). Experiment C also gave similar responses, generating a higher survival for VD (56%) than CTA (52%) and abdominal muscle (49%).

Melanized implants exhibited a dark coloration covering parts or the whole implant. Normal implants showed white coloration, without any trace of melanization (Fig. 1). One week after implantation, females from Experiment A showed a larger fraction of melanized implants (DTA-AG: 12/47; Control-PTA: 6/51) in comparison with Experiment B females (CTA-AG: 2/43; Control-VD: 1/34; Table 1). Histology of melanized implants showed necrotic tissue, characterized by the absence of normal cellular arrangement, with no definition of tissue layers, containing few or no nuclei and undistinguishable cell boundaries, encapsulated by black depositions. These melanized capsules were surrounded externally by normal haemocytes and flat haemocytes in the middle (Fig. 2). Some implants showed melanized capsules with

necrotic tissue inside, and apparently viable non-necrotic tissue outside the capsule (Fig. 2C and D).

Grafts from Experiment C (CTA-AG, VD and M) recovered at day 7 and 15, showed a gradual decrease in size. No differences were observed between male and female recipients. However, M implants exhibited a higher rate of tissue size-reduction compared to CTA-AG and VD grafts. Size differences between day 1 and 15 were statistically significant for CTA-AG ( $t = 5.20$ , 47 d.f.,  $p < 0.05$ ), VD ( $t = 4.12$ , 8 d.f.,  $p < 0.05$ ) and M ( $t = 9.05$ , 4.35 d.f.,  $p < 0.05$ ) (Fig. 3). Despite the size-reduction of grafts, their external shape did not show evident changes from control tissues (Fig. 4).

Histology of grafts from Experiment C (Fig. 5) indicated that tissue remained viable 7 and 15 days post implantation. The cellular structure of tissues was similar to control tissues 15 days post implantation for terminal ampoules and vasa deferentia. These organs maintained a normal tissue configuration as well as a normal cell structure, without any encapsulation-melanization sign, indicating the viability of implants. Abdominal muscle implants with a faster rate of size reduction, showed a less dense muscle fibre configuration than control muscle sections 7 days post implantation.

Implant recovery rates from Experiment C were higher for ampoules (31/33) than vasa deferentia (24/34) and abdominal muscle (15/23). At day 7, 4 implants (2 M and 2 VD) were undetected, whereas at day 15, 16 implants (8 VD, 6 M and 2 CTA-AG) were undetected. Those grafts were classified as missing after a detailed unsuccessful search in the implantation region and adjacent areas where the implant could have moved.

### 3.2. Expression of primary and secondary sex characters

Bilaterally eyestalk ablated males (15 days) developed hypertrophied AGs as compared to AGs from normal males. Presumptive AG cells were observed in cross sections of terminal ampoules of normal young males (B.W. = 9.32 g). Histological observation showed a massive homogeneous group of cells at the distal section of the terminal ampoules which were organized in grape-like clusters (Fig. 6).

At the end of Experiment A, DTA-AG females were bigger ( $t = 2.19$ , 39 d.f.,  $p < 0.05$ ) and heavier ( $t = 3.49$ , 39 d.f.,  $p < 0.05$ ) than Control-PTA females, generating around 1.0 g difference. However, those groups were cultured in separated tanks. On the other hand, females from

**Table 1**  
Evaluation of male reproductive tissue implantation in *Litopenaeus vannamei* females.

Parameter	Experiment A		Experiment B		
	DTA-AG	Control-PTA	CTA-AG	Control-VD	Control I <sup>1</sup>
Initial number of females	47	51	43	34	32
Number of survivors (1 h)	35 (74%)	41 (80%)	29 (67%)	28 (82%)	31 (97%)
Number of survivors (24 h)	33 (70%)	41 (80%)	28 (65%)	27 (79%)	31 (97%)
Number of survivors (7 days)	30 (64%)	39 (76%)	24 (56%)	25 (74%)	26 (81%)
Number of final survivors	15 (32%)	28 (55%)	21 (49%)	15 (44%)	19 (60%)
Melanized implants (7 days)	12	6	2	1	–
Melanized implants (final number)	2	0	2	1	–
Initial age	PL <sub>34</sub> , PL <sub>40</sub> , PL <sub>48</sub>	PL <sub>34</sub> , PL <sub>40</sub> , PL <sub>48</sub>	PL <sub>37</sub>	PL <sub>37</sub>	PL <sub>37</sub>
Culture time (days)	63, 71, 78	63, 71, 78	84	84	84
Initial size (mm)	58.54 ± 2.50 <sup>a</sup>	57.7 ± 2.39 <sup>a</sup>	40.98 ± 5.39 <sup>a</sup>	41.10 ± 5.17 <sup>a</sup>	38.30 ± 3.83 <sup>a</sup>
Initial weight (g)	2.86 ± 0.39 <sup>a</sup>	2.71 ± 0.37 <sup>a</sup>	1.05 ± 0.45 <sup>a</sup>	1.03 ± 0.42 <sup>ab</sup>	0.81 ± 0.27 <sup>b</sup>
Final size (mm)	83.24 ± 3.71 <sup>a</sup>	80.74 ± 3.26 <sup>b</sup>	59.97 ± 6.57 <sup>a</sup>	56.03 ± 9.52 <sup>ab</sup>	53.86 ± 5.97 <sup>b</sup>
Final weight (g)	8.68 ± 1.18 <sup>a</sup>	7.48 ± 0.95 <sup>b</sup>	3.07 ± 1.10 <sup>a</sup>	2.64 ± 1.23 <sup>a</sup>	2.42 ± 0.92 <sup>a</sup>
L.E.L./T.L. <sup>2</sup>	0.0272 ± 0.0076 <sup>a</sup>	0.0245 ± 0.0026 <sup>a</sup>	0.0216 ± 0.0026 <sup>a</sup>	0.0220 ± 0.0027 <sup>a</sup>	0.0218 ± 0.0027 <sup>a</sup>
R.E.L./T.L. <sup>3</sup>	0.0208 ± 0.0020 <sup>a</sup>	0.0207 ± 0.0012 <sup>a</sup>	0.0199 ± 0.0025 <sup>a</sup>	0.0198 ± 0.0019 <sup>a</sup>	0.0206 ± 0.0022 <sup>a</sup>
Size (mm) at 192 days of culture <sup>4</sup>			73.08 ± 8.02 <sup>a</sup>	68.72 ± 9.56 <sup>a</sup>	72.15 ± 8.41 <sup>a</sup>
Weight (g) at 192 days of culture <sup>4</sup>			5.78 ± 1.83 <sup>a</sup>	4.93 ± 1.95 <sup>a</sup>	5.43 ± 1.80 <sup>a</sup>
L.E.L./T.L. (192 days) <sup>4</sup>			0.0242 ± 0.003 <sup>a</sup>	0.0212 ± 0.007 <sup>a</sup>	0.0240 ± 0.003 <sup>a</sup>

Means with different letters between treatments are statistically different ( $p < 0.05$ ).

Distal terminal ampoule with androgenic gland (DTA-AG), proximal terminal ampoule (PTA), complete terminal ampoule (CTA-AG), vas deferens (VD), intact control (Control I).

<sup>1</sup> Control I did not receive any implant, only the amputation of the first left pleopod.

<sup>2</sup> Normalized left first endopodite length.

<sup>3</sup> Normalized right first endopodite length.

<sup>4</sup> Additional culture period (only for Group B).

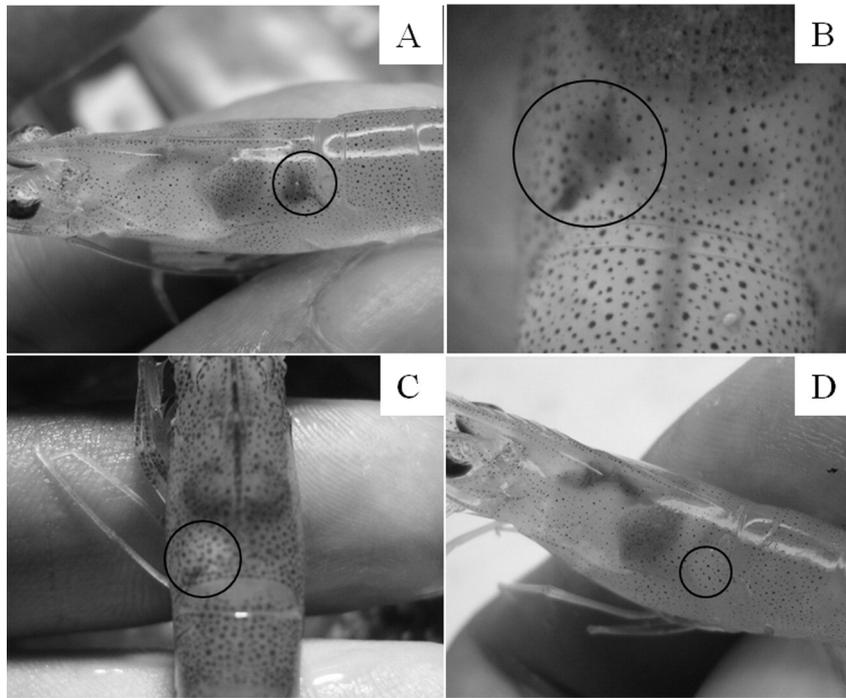


Fig. 1. Appearance of melanized (A and B), and normal (C and D) implants 7 days post implantation. Black circles show the implant position.

Experiment B showed higher length ( $F_{2,50} = 3.37$ ,  $p < 0.05$ ) for CTA-AG implanted as compared to Intact Control at 84 days. Nonetheless, at day 192 (extra culture period), the three groups showed a similar size and weight. Normalized length of first left endopodite (L.E.L./T.L.) and right endopodite (R.E.L./T.L.) were similar between controls and AG implanted females in both groups (A and B; Table 1).

*Appendices masculina* did not develop in any of the implanted females or controls. Regenerated endopodites grew larger in comparison with endopodites that remained intact. This pattern was observed in all treatments, however was more evident in females from group A.

Concerning petasma development, one of the females implanted with AG showed a straight shape at the distal region and a middle protuberance in the regenerated first endopodite (Fig. 7A–B). Another female showed a regenerated first endopodite that was twice as long as the intact endopodite, which also showed a straight distal shape and a middle protuberance (Fig. 7C–D). The regenerated endopodite of the former female and the intact endopodite of the later female did not develop setae along the middle curved edge of the structure (Fig. 7A–C). These setae are typical of normal females. In some AG implanted females, although this pattern was present, it was less pronounced (Fig. 7E–F). In all these cases, the regenerated endopodite showed a different shape than normal endopodite in females. Regenerated and intact first endopodites of Control-PTA females showed a slender shape with a curved distal edge, without a middle protuberance and the characteristic setae along the middle curved edge of endopodites.

Females from group A showed differences in the pattern of setae distribution. In DTA-AG implanted females, 36% of the regenerated endopodites showed some setae at the middle curved edge of the structure, whereas in Control-PTA females, 75% of endopodites exhibited the normal pattern of setae in this region. Pattern of setae distribution did not show any difference in females from group B.

Oocytes from females implanted with AG (DTA-AG,  $n = 5$ ) were found at the same stage of development as in control females (Control-PTA,  $n = 2$ ). Histological observation showed oocytes at a previtellogenic phase, most of them at the early perinucleolus phase, with a size  $< 50 \mu\text{m}$ , and a cytoplasm with relatively low volume related to the nucleus. There was no evidence of masculinization or abnormal development of oocytes in AG implanted females (Fig. 8).

## 4. Discussion

### 4.1. State of implants

The present study demonstrates for the first time in the genus *Litopenaeus* that intraspecific reproductive tissue grafts are immunologically accepted maintaining their viable condition. This response follows the accepted knowledge from Arthropoda, including other Crustacea, in the sense that intraspecific transplants do not provoke encapsulation (Lackie, 1986). The evidence presented here and previously by Alfaro et al. (2009), also may suggest graft absorption in relation to tissue composition. Terminal ampoules and vasa deferentia tissues were less affected than muscle and nerve cord-ganglia tissues. Intraspecific grafted AG tissues from other decapods have been active inducing sex reversal (Nagamine et al., 1980b; Malecha et al., 1992; Karplus et al., 2003; Manor et al., 2004). In case AG grafts are indeed absorbed, it might be a constraint for the prolonged hormonal effect of AG implants; nevertheless, some minor masculinizing effects were measured on body growth and petasma formation in the present study.

Previous studies of AG grafting in the freshwater prawn *M. rosenbergii* generated survival rates from 10.5% (Malecha et al., 1992) to 100% (Nagamine et al., 1980b). Survival rates in other malacostracan crustaceans have fluctuated from 48% in the decapod *Procambarus clarkii* (Taketomi and Nishikawa, 1996) to 90% in the isopod *Armadillidium vulgare* (Suzuki and Yamasaki, 1997). In *L. vannamei* males, surgery for AG removal gave 76% survival at 69 mm T.L. and 26% at 37 mm T.L. (Alfaro-Montoya et al., 2015). Therefore, the survival rates measured in the present study (32 and 49%) seem comparable to previous studies. Differences between survival rates of control implants versus implants containing AGs may be influenced by collateral damages caused by the size of implanted tissue, being of smaller size for proximal terminal ampoules (PTA) and vasa deferentia (VD).

Most implants investigated in the present study exhibited intact cellular appearance similar to thoracic-nerve cord implants studied previously (Alfaro et al., 2009). However, few complete or partial melanization cases were found in the present study. As in arthropods, such encapsulation and nodule formation phenomena are triggered as part of a rejection mechanism against foreign tissues (Lackie, 1986)

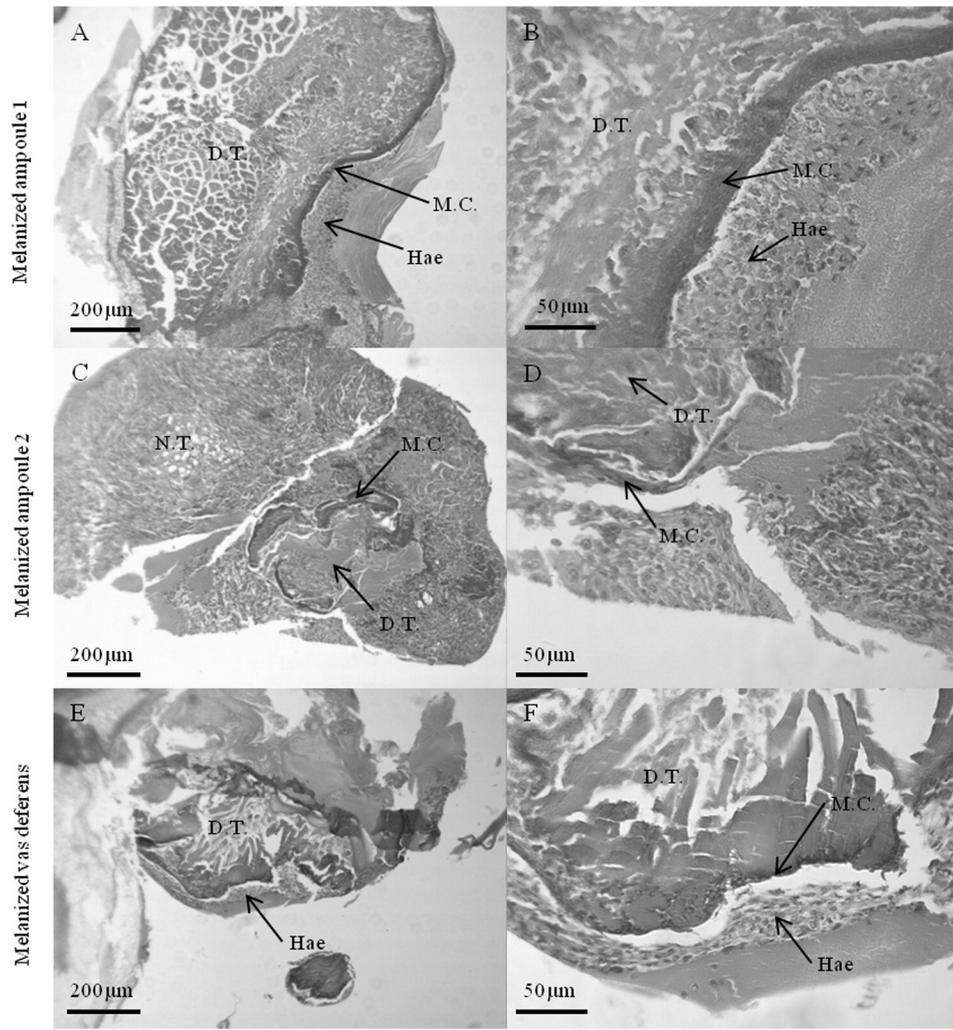


Fig. 2. Histology of melanized implants, 7 days post implantation. D.T. = deteriorated tissue, M.C. = melanized capsule, Hae = haemocyte, N.T. = normal tissue.

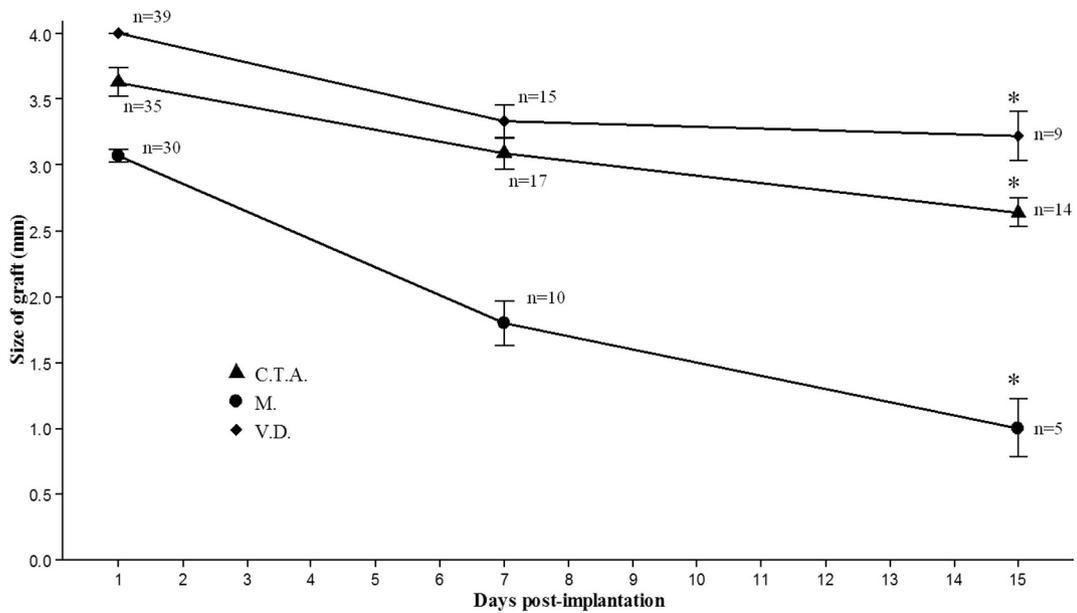
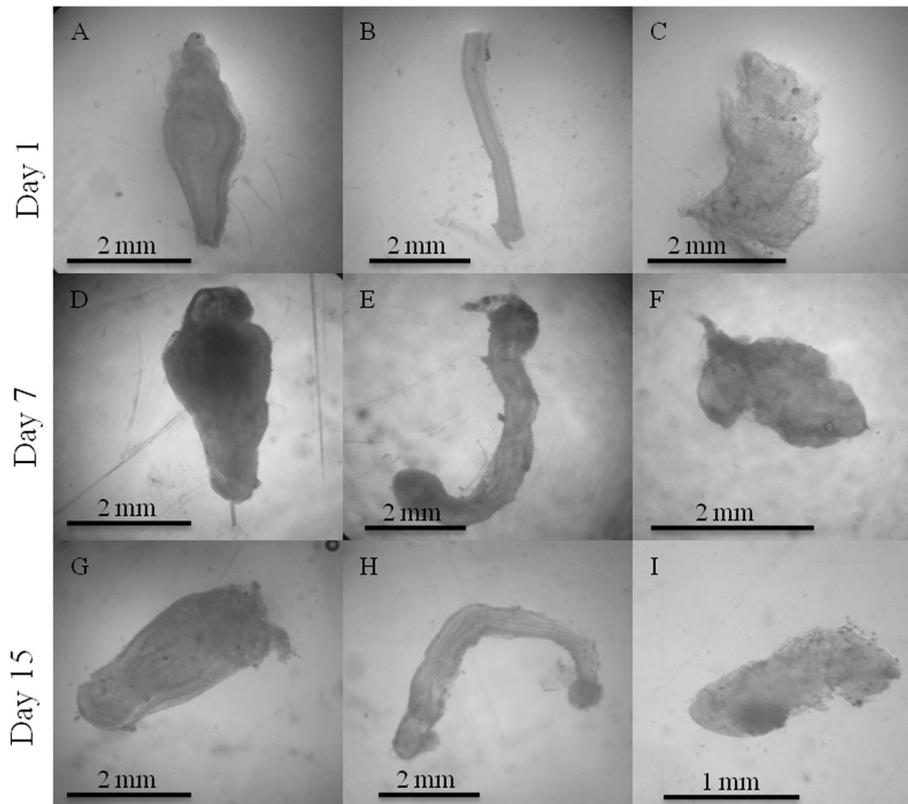
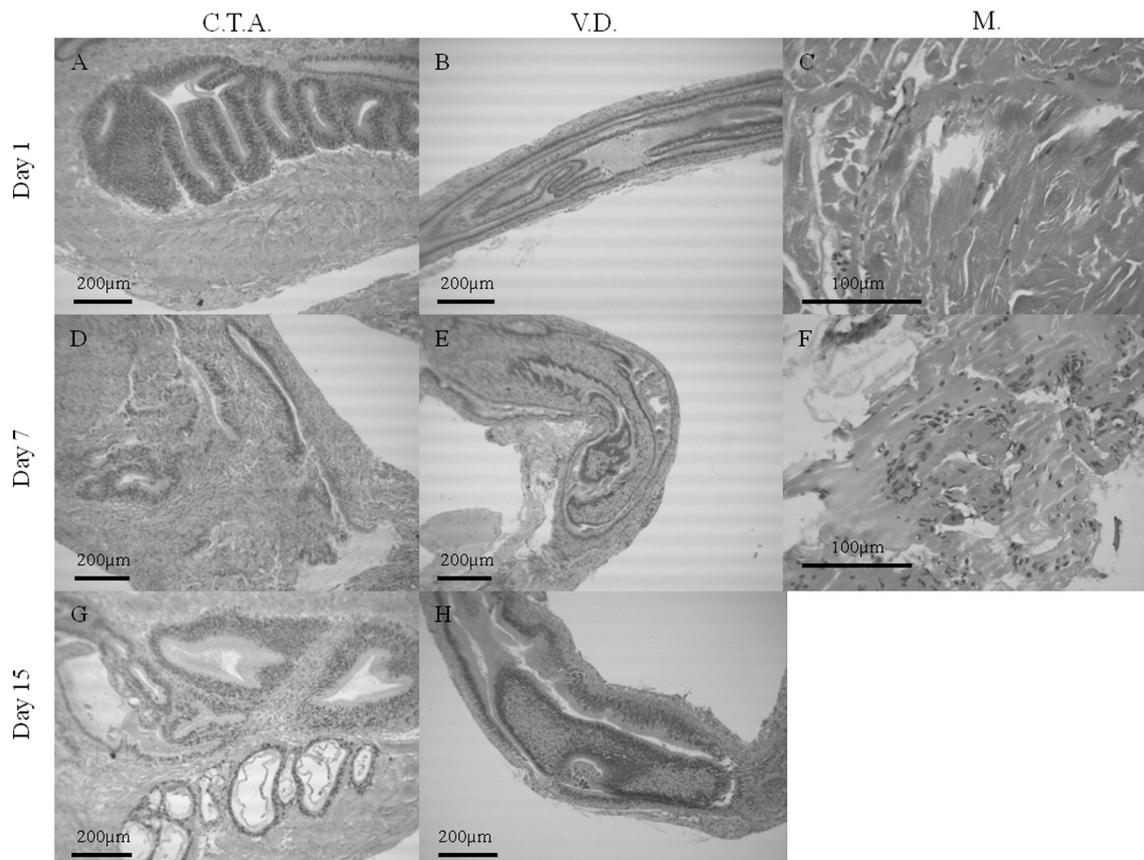


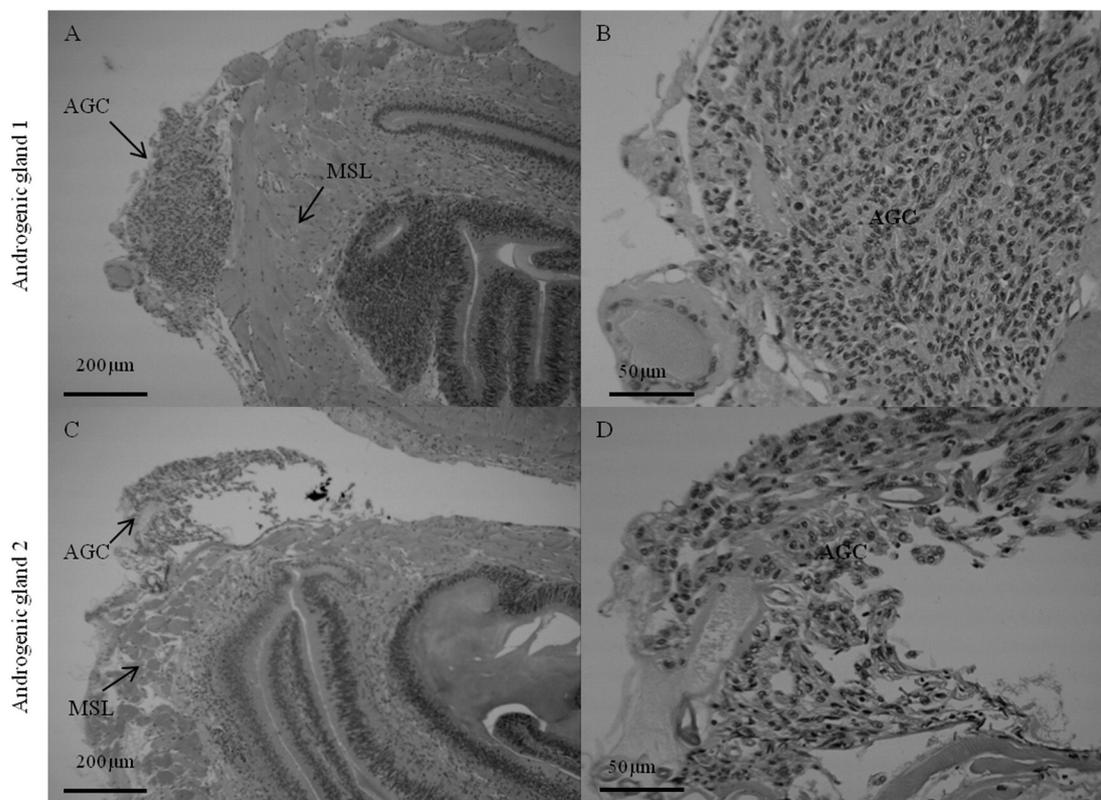
Fig. 3. Reduction of graft size, 7 and 15 days post implantation. Vertical bars indicate standard error. \* Represent significant difference in size ( $p < 0.05$ ) between day 1 and 15. C.T.A. = complete terminal ampoule, V.D. = vas deferens, M. = abdominal muscle.



**Fig. 4.** General morphology of accepted grafts at 1, 7 and 15 days post implantation. Complete terminal ampoule (A, D and G), vas deferens (B, E and H), abdominal muscle (C, F and I).



**Fig. 5.** Histology of implants, at 1, 7 and 15 days post implantation. C.T.A. = complete terminal ampoule, V.D. = vas deferens, M. = abdominal muscle.



**Fig. 6.** Histology of terminal ampoule from donors (B.W. = 9.32 g), showing presumptive androgenic gland cells. Ampoule 1 (A and B) and ampoule 2 (C and D). AGC = androgenic gland cells, MSL = muscle layer.

and infectious pathogens (Shinn-Pyng et al., 2009). Crustacean haemocytes are involved in the immune response including recognition, phagocytosis, melanization, cytotoxicity and cell–cell communication (Shinn-Pyng et al., 2009). The phenoloxidase enzymatic system from granular and semi granular haemocytes is responsible for the melanization of foreign objects and pathogens in crustaceans including penaeid shrimps (Ashida and Söderhäll, 1984; Söderhäll and Smith, 1986; Tsing et al., 1989; Sritunyalucksana and Söderhäll, 2000). Histological analysis of the few melanized implants found in the present study clearly showed deteriorated tissue residues inside capsules; capsules are integrated by an inner layer of necrotic haemocytes with black depositions, surrounded by separated layers of flat and normal haemocytes as found previously by Alfaro et al. (2009).

Similar immunological responses were measured by Alfaro et al. (2009), who implanted intraspecific and interspecific thoracic nerve cord–ganglia sections into *L. vannamei*. The study found low tissue rejection within the family Penaeidae, and a high rate of encapsulation-melanization in implants from *Macrobrachium tenellum* (Palaemonidae). These two studies in Penaeidae support the accepted knowledge from Arthropoda concerning tissue recognition and the phylogenetic closeness between donor and recipient (Lackie, 1986).

The reduction in the size of grafts measured in the present study was more evident for abdominal muscle than ampoules and vasa deferentia, complementing the previous findings described by Alfaro et al. (2009) about size-reduction of thoracic nerve-ganglia grafts in *Litopenaeus stylirostris*. The presence and composition of an external connective tissue layer around grafts seem to affect the haemocytic response in arthropods (Lackie, 1986), however, no similar absorption mechanism has been described from other crustaceans. Total missing grafts in the present study were similar to observations about missing implants reported by Nagamine et al. (1980b) and Alfaro et al. (2009). Possible explanations for such losses are graft expulsion through molting or complete degradation and absorption. Ampoules and vasa deferentia implants seem to be accepted by the host, presumably maintaining

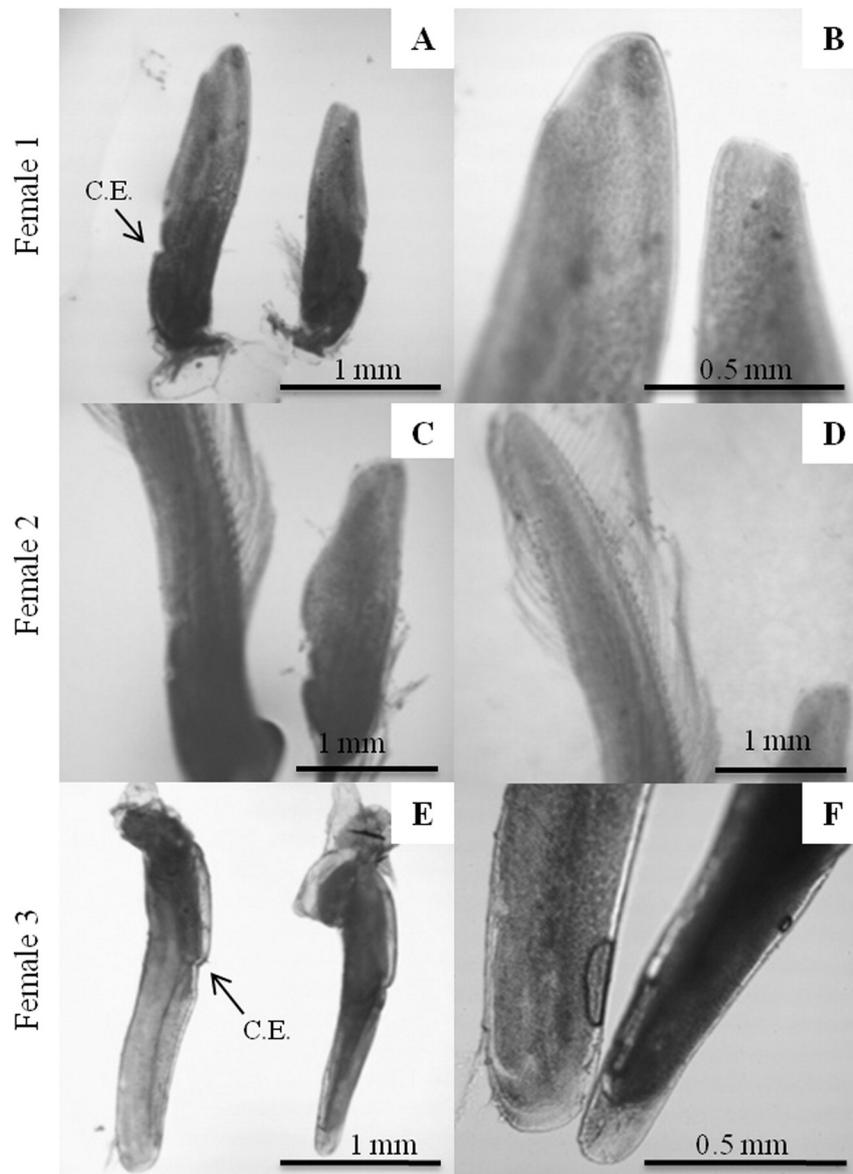
cellular activity; whether androgenic hormone synthesis from implants is active requires further confirmation.

An absorption mechanism of grafted tissue has been described for annelids (Götz, 1986; Hostetter and Cooper, 1972; Linthicum et al., 1977). Hostetter and Cooper (1972) implanted tissues from *Eisenia foetida* into *Lumbricus terrestris*, finding that grafts were invaded by two types of coelomocytes that phagocytized differentially the connective cells and muscle fibers. Linthicum et al. (1977) reported from *L. terrestris*, that tissues after 11–13 days of implantation had lost their muscle cells by the phagocytic activity of coelomocytes. In *Litopenaeus*, there is evidence of an alternative mechanism for intraspecific graft elimination through absorption, possibly as a natural healing process by the host to restore continuity with self-recognized tissues (Lackie, 1986; Alfaro et al., 2009); therefore, a similar mechanism may be operating in crustaceans, and this should be further studied.

#### 4.2. Effects on primary and secondary characters

The presumptive AG cells observed in this study are comparable to previous reports (Campos-Ramos et al., 2006; Garza-Torres et al., 2009; Alfaro-Montoya and Hernández, 2012). The AG in young males (B.W. = 9 g) is located at the distal regions of the terminal ampoule as reported by Vázquez-Islas et al. (2014) and Alfaro-Montoya et al. (2015) for adults (B.W. = 24–30 g). Thus it seems that AG tissue was indeed implanted despite the minor observable effects on sexual characters.

In *L. vannamei* (Alfaro-Montoya et al., 2015), *C. quadricarinatus* (Manor et al., 2004) and *M. rosenbergii* (Sagi et al., 1990; Sagi and Cohen, 1990), the AG has been linked with somatic growth enhancement in males. However, in penaeids, females grow larger after reaching 15–20 g (Gopal et al., 2010). In this study, size differences were observed only between CTA-AG and Control at day 84 post-implantation, while at day 192 no differences in size or weight were detected between those groups.



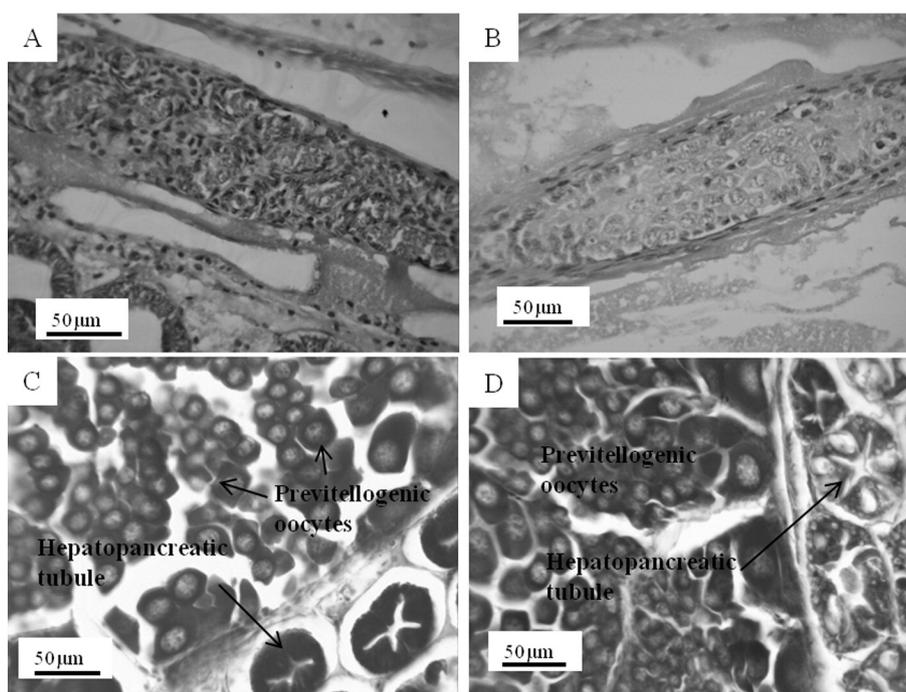
**Fig. 7.** Endopodites of first pleopods from three females implanted with distal terminal ampoule (DTA-AG). Regenerated (left) and intact (right) endopodites. C.E.: curved edge without setae.

Unlike previous reports on effects of AG removal and implantation in crustaceans on external secondary sex characters (Nagamine et al., 1980b; Malecha et al., 1992; Karplus et al., 2003; Manor et al., 2004; Alfaro-Montoya et al., 2015), the only effects of implantations found in the present study were abnormal development of first pleopod endopodites in two DTA-AG implanted females. Additionally, a lower percentage of the regenerated endopodites was observed in DTA-AG females with some setae at the curved edge of the structure. These shapes and setae distribution patterns are not observed in endopodites of normal females (Campos-Ramos et al., 2006) suggesting possible effect of AG implantation on normal development of these structures.

Development of oocytes seemed normal in DTA-AG implanted females. The oocytes seemed previtelogenic in an early perinucleolus phase (Alfaro-Montoya, 2013). Therefore, AG implantation did not induce any measurable effect on gonad development during the 2–3 months of experimentation. Garza-Torres (2006) andrectomized *L. vannamei* males (15–18 g) without detecting any sign of external/internal sex change. However, working on smaller males ( $4.36 \pm 1.44$  g), Alfaro-Montoya et al. (2015) detected differences in body, petasma and *appendix masculina* size following andrectomy. Spermatogenic

alterations were also observed in testis after 12 months of culture, indicating that spermatid maturation was stopped and cellular degradation was activated due to the removal of the AG.

Previous reports have demonstrated that the degree of masculinization could differ between species and between individuals of the same species. For instance, in *M. rosenbergii*, some degree of masculinization was shown in AG implanted females regardless the developmental stage at implantation, however complete masculinization and spermatogenesis were observed only in females implanted at very early developmental stage (Nagamine et al., 1980b). Malecha et al. (1992) observed that while all AG implanted *M. rosenbergii* females showed some degree of masculinization, less than half were able to mate as males. The authors related this result with the developmental stage at implantation. Similar responses were observed in *C. quadricarinatus* (Khalaila et al., 2001; Karplus et al., 2003; Manor et al., 2004). However, despite the differences in the degree of masculinization between individuals, in all previous cases masculinization was evident. Contrarily, in the present study only some abnormalities were observed in the development of endopodites of the first pair of pleopods of DTA-AG females, suggesting a possible effect of AG implantation, however, no



**Fig. 8.** Histology of normally developed gonads of juvenile male (A), juvenile females (B), control female (C, B.W. = 7.5 g) and a representative distal terminal ampoule (DTA-AG) implanted female (D, B.W. = 8.7 g).

conclusive evidence of masculinization was found. These results could be related to the age and size at implantation. According to Garza-Torres et al. (2009) processes of sex differentiation in *L. vannamei* begin at PL<sub>12</sub> while its external exhibition starts around PL<sub>32</sub>. For that reason, it is considered that the AG implanted females in the present study were in an advanced sexual stage. This is supported by numerous previous studies which demonstrated a clear relationship between the degree of masculinization and the age or size of females at AG implantation (Nagamine et al., 1980b; Taketomi and Nishikawa, 1996; Fowler and Leonard, 1999; Manor et al., 2004; Aflalo et al., 2006). Additionally, Nagamine et al. (1980a, 1980b) found an inverse relationship between the age of andrectomy and the degree of feminization.

Based on the results of the present study, we suggest testing AG implantation on younger females. As suggested by previous researchers this could be done using hypertrophied AG (Alfaro-Montoya et al., 2015; Khalaila et al., 2001), AG cells (Levy et al., 2016) or even AG extracts (Malecha and Sun, 2004; Malecha, 2012) or recombinant IAG (Aizen et al., 2016). The knowledge accumulated with respect to sexual plasticity of decapod crustaceans thus far comes from few species of the suborder Pleocyemata (Aflalo et al., 2012; Rungsin et al., 2006; Aflalo et al., 2006; Manor et al., 2004; Fowler and Leonard, 1999) and Oniscidea (Suzuki et al., 1990; Suzuki and Yamasaki, 1997). Sexual plasticity is yet to be demonstrated in the family Penaeidae (suborder Dendrobranchiata).

In summary, reproductive intraspecific transplants in *L. vannamei* were in most cases accepted, and an absorption mechanism might be activated depending on tissue structure. The absorption of grafts seems to be a slow process for connective tissue coated implants, allowing the grafts to be active (e.g. IAG secretion) for a certain period of time since some minor evidence of AG activity in females were detected. However, sexual plasticity in Penaeidae still requires confirmation through the use of younger females (PL<sub>12</sub>) and novel hormone delivery approaches.

#### Acknowledgements

This research is part of the project: Sex reversal of the cultured marine shrimp, *Litopenaeus vannamei*, and the post-graduate studies of Luis

Vega. It was financially supported by Consejo Nacional de Rectores (CONARE), Costa Rica, and was scientifically supported by Ben-Gurion University of the Negev, Israel. The authors wish to thank shrimp farmers from Golfo de Nicoya, Jose Valverde Moya and Jorge Paternina Mancilla (Laboratorio de Larvicultura Activos de Crustáceos del Pacífico S.A.) for their cooperation in shrimp postlarvae supply.

#### References

- Aflalo, E.D., Hoang, T.T., Nguyen, V.H., Lam, Q., Nguyen, D.M., Trinh, Q.S., Raviv, S., Sagi, A., 2006. A novel two-step procedure for mass production of all-male populations of the giant freshwater prawn *Macrobrachium rosenbergii*. *Aquaculture* 256 (1), 468–478.
- Aflalo, E.D., Raju, D.V., Bommi, N.A., Verghese, J.T., Samraj, T.Y., Hulata, G., Ovadia, O., Sagi, A., 2012. Toward a sustainable production of genetically improved all-male prawn (*Macrobrachium rosenbergii*): evaluation of production traits and obtaining neo-females in three Indian strains. *Aquaculture* 338, 197–207.
- Aizen, J., Chandler, J.C., Fitzgibbon, Q.P., Sagi, A., Battaglione, S.C., Elizur, A., Ventura, T., 2016. Production of recombinant insulin-like androgenic gland hormones from three decapod species: in vitro testicular phosphorylation and activation of a newly identified tyrosine kinase receptor from the Eastern spiny lobster, *Sagmariasus verreauxi*. *Gen. Comp. Endocrinol.* <http://dx.doi.org/10.1016/j.ygcen.2016.02.013>.
- Alfaro, J., Hernández, L., Zúñiga, G., Soto, R., Mejía-Arana, F., 2009. Thoracic nerve cord-ganglia recognition in intraspecific and interspecific transplants in the white shrimp, *Penaeus (Litopenaeus) vannamei*. *Aquaculture* 288, 126–131.
- Alfaro-Montoya, J., 2013. Descripción histológica de la ovogénesis y espermatogénesis del camarón de cultivo, *Litopenaeus vannamei*. *Rev. Biol. Mar. Oceanogr.* 48 (2), 335–344.
- Alfaro-Montoya, J., Hernández, L., 2012. The histological structure of the androgenic gland and cellular cord of the male reproductive system of adult *Litopenaeus* and *Rimapenaeus byrdi*. *J. Crust. Biol.* 32, 351–357.
- Alfaro-Montoya, J., Hernández-Noguera, L., Vega-Alpizar, L., Umaña-Castro, R., 2015. Effects of androgenic gland ablation on growth, sexual characters and spermatogenesis of the white shrimp, *Litopenaeus vannamei* (Decapoda: Penaeidae) males. *Aquacult. Res.* <http://dx.doi.org/10.1111/are.12727>.
- Ashida, M., Söderhäll, K., 1984. The prophenoloxidase activating system in crayfish. *Comp. Biochem. Physiol.* 77B, 21–26.
- Bell, T.A., Lightner, D.V., 1988. *A Handbook of Normal Penaeid Shrimp Histology*. World Aquaculture Society, Baton Rouge, Louisiana 114 pp.
- Benzie, J.A., Kenway, M., Ballment, E., 2001. Growth of *Penaeus monodon* X *Penaeus esculentus* tiger prawn hybrids relative to the parental species. *Aquaculture* 193, 227–237.
- Campos-Ramos, R., Garza-Torres, R., Guerrero-Tortolero, D.A., Maeda-Martínez, A.M., Obregón-Barboza, H., 2006. Environmental sex determination, external sex differentiation and structure of the androgenic gland in the Pacific white shrimp *Litopenaeus vannamei* (Boone). *Aquac. Res.* 37, 1583–1593.
- Charniaux-Cotton, H., 1954. Découverte chez un Crustacé Amphipode (*Orchestia gammarella*) d'une glande endocrine responsable de la différenciation des caractères sexuels primaires et secondaires males. *CR Acad. Sci.* 239, 780–782.

- R Core Team, 2014. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria <http://www.R-project.org/>.
- Cronin, L.E., 1947. Anatomy and histology of the male reproductive system of *Callinectes sapidus* Rathbun. *J. Morphol.* 81 (2), 209–239.
- Fowler, R.J., Leonard, B.V., 1999. The structure and function of the androgenic gland in *Cherax destructor* (Decapoda: Parastacidae). *Aquaculture* 171, 135–148.
- Garza-Torres, R., 2006. Determinación sexual medioambiental, diferenciación sexual externa y estructura de la glándula androgénica en el camarón blanco del pacífico *Litopenaeus vannamei* (Decapoda: Penaeidae). Tesis de Maestría. Centro de Investigaciones Biológicas del Noreste, S.C., La Paz, México.
- Garza-Torres, R., Campos-Ramos, R., Maeda-Martínez, A.M., 2009. Organogenesis and subsequent development of the genital organs in female and male Pacific white shrimp *Penaeus (Litopenaeus) vannamei*. *Aquaculture* 296, 136–142.
- Gopal, C., Gopikrishna, G., Krishna, G., Jahageerdar, S.S., Rye, M., Hayes, B.J., Paulpandi, S., Kiran, R.P., Pillai, S.M., Ravichandran, P., Ponniah, A.G., Kumar, D., 2010. Weight and time of onset of female-superior sexual dimorphism in pond reared *Penaeus monodon*. *Aquaculture* 300, 237–239.
- Götz, P., 1986. Encapsulation in arthropods. In: Brehelin, M. (Ed.), *Immunity in Invertebrates: Cells, Molecules and Defense Reactions*. Springer-Verlag, Berlin, pp. 153–170.
- Hostetter, R.K., Cooper, E.L., 1972. Coelomocytes as effector cells in earthworm immunity. *Immunol. Inves.* 1 (2), 155–183.
- Karplus, I., Sagi, A., Khalaila, I., Barki, A., 2003. The influence of androgenic gland implantation on the agonistic behavior of female crayfish (*Cherax quadricarinatus*) in interaction with males. *Behaviour* 140, 649–663.
- Khalaila, I., Katz, T., Abdu, U., Yehezkel, G., Sagi, A., 2001. Effects of implantation of hypertrophied androgenic glands on sexual characters and physiology of the reproductive system in the female red claw crayfish, *Cherax quadricarinatus*. *Gen. Comp. Endocrinol.* 121 (3), 242–249.
- Lackie, A.M., 1986. Transplantation immunity in arthropods: is immune recognition merely wound-healing. In: Brehelin, M. (Ed.), *Immunity in Invertebrates*. Springer-Verlag, New York, USA, pp. 125–138.
- Levy, T., Rosen, O., Eilam, B., Azuly, D., Aflalo, E.D., Manor, R., Shechter, A., Sagi, A., 2016. A single injection of hypertrophied androgenic gland cells produces all-female aquaculture. *Mar. Biotechnol.* doi: <http://dx.doi.org/10.1007/s10126-016-9717-5>.
- Linthicum, D.S., Marks, D.H., Stein, E.A., Cooper, E.L., 1977. Graft rejection in earthworms: an electron microscopic study. *Eur. J. Immunol.* 7 (12), 871–876.
- Malecha, S.R., 2012. The case for all-female freshwater prawn, *Macrobrachium rosenbergii* (De Man), culture. *Aquac. Res.* 43, 1038–1048.
- Malecha, S.R., Sun, P.S., 2004. Methods of isolating the androgenic sex hormone from crustacean and marine shrimp and methods of use. US Patent No. 6,740,794 B1 issued 25 May 2004. United States Patent Office, Available at <http://www.uspto.gov/patents/process/search/> (accessed 11 October 2011).
- Malecha, S.R., Nevinb, P.A., Haa, P., Barckb, L.E., Lamadrid-Roseb, Y., Masunob, S., Hedgecock, D., 1992. Sex-ratios and sex-determination in progeny from crosses of surgically sex-reversed freshwater prawns, *Macrobrachium rosenbergii*. *Aquaculture* 105, 201–218.
- Manor, R., Aflalo, E.D., Segall, C., Weil, S., Azuly, D., Ventura, T., Sagi, A., 2004. Androgenic gland implantation promotes growth and inhibits vitellogenesis in *Cherax quadricarinatus* females held in individual compartments. *Invertebr. Repr. Dev.* 45 (2), 151–159.
- Nagamine, C., Knight, A.W., Maggenti, A., Paxman, G., 1980a. Effects of androgenic gland ablation on male primary and secondary sexual characteristics in the Malaysian prawn, *Macrobrachium rosenbergii* (de Man) (Decapoda, Palaemonidae), with first evidence of induced feminization in a nonhermaphroditic decapod. *Gen. Comp. Endocrinol.* 41 (4), 423–441.
- Nagamine, C., Knight, A.W., Maggenti, A., Paxman, G., 1980b. Masculinization of female *Macrobrachium rosenbergii* (de Man) (Decapoda, Palaemonidae) by androgenic gland implantation. *Gen. Comp. Endocrinol.* 41 (4), 442–457.
- Ro, S., Talbot, P., Leung-Trujillo, J., Lawrence, A.L., 1990. Structure and Function of the vas deferens in the shrimp *Penaeus setiferus*: segments 1–3. *J. Crust. Biol.* 10, 455–468.
- Robinson, N.A., Gopikrishna, G., Baranski, M., Kumar-Katneni, V., Shekhar, M.S., Shanmugakarthik, J., Jothivel, S., Gopal, C., Ravichandran, P., Gitterle, T., Ponniah, A.G., 2014. QTL for white spot syndrome virus resistance and the sex-determining locus in the Indian black tiger shrimp (*Penaeus monodon*). *BMC Genomics* 15, 731.
- Rungsin, W., Paankhao, N., Na-Nakorn, U., 2006. Production of all-male stock by neofemale technology of the Thai strain of freshwater prawn, *Macrobrachium rosenbergii*. *Aquaculture* 259, 88–94.
- Sagi, A., Cohen, D., 1990. Growth, maturation and progeny of sex-reversed *Macrobrachium rosenbergii* males. *World Aquaculture* 21 (4), 87–90.
- Sagi, A., Cohen, D., Milner, Y., 1990. Effect of androgenic gland ablation on morphotypic differentiation and sexual characteristics of male freshwater prawns, *Macrobrachium rosenbergii*. *Gen. Comp. Endocrinol.* 77, 15–22.
- Shinn-Pyng, Y., Ying-Nan, C., Shu-Ling, H., Winton, C., Chun-Hung, L., 2009. Immune response of white shrimp, *Litopenaeus vannamei*, after a concurrent infection with white spot syndrome virus and infectious hypodermal and hematopoietic necrosis virus. *Fish Shellfish Immunol.* 26, 582–588.
- Söderhäll, K., Smith, V.J., 1986. The prophenoloxidase activating system: the biochemistry of its activation and role in arthropod cellular immunity, with special reference to crustaceans. In: Brehelin, M. (Ed.), *Immunity in Invertebrates*. Springer-Verlag, New York, USA, pp. 208–223.
- Sritunyalucksana, K., Söderhäll, K., 2000. The proPO and clotting system in crustaceans. *Aquaculture* 191, 53–69.
- Staelens, J., Rombaut, D., Vercauteren, I., Argue, B., Benzie, J., Vuylsteke, M., 2008. High-density linkage maps and sex-linked markers for the black tiger shrimp (*Penaeus monodon*). *Genetics* 179 (2), 917–925.
- Suzuki, S., Yamasaki, K., 1997. Sexual bipotentiality of developing ovaries in the terrestrial isopod *Armadillidium vulgare* (Malacostraca, Crustacea). *Gen. Comp. Endocrinol.* 107 (1), 136–146.
- Suzuki, S., Yamasaki, K., Katakura, Y., 1990. Vitellogenin synthesis in andrectomized males of the terrestrial isopod, *Armadillidium vulgare* (Malacostracan Crustacea). *Gen. Comp. Endocrinol.* 77 (2), 283–291.
- Taketomi, Y., Nishikawa, S., 1996. Implantation of androgenic glands into immature female crayfish, *Procambarus clarkii*, with masculinization of sexual characteristics. *J. Crust. Biol.* 16 (2), 232–239.
- Tsing, A., Arcier, J., Brehelin, M., 1989. Hemocytes of penaeid and palaemonid shrimps: morphology, cytochemistry, and hemograms. *J. Invertebr. Pathol.* 53, 64–77.
- Vázquez-Islas, G., Garza-Torres, R., Guerrero-Tortolero, D., Campos-Ramos, R., 2014. Histology of the androgenic gland and expression of the insulin-like androgenic gland hormone precursor gene in the genital organ of Pacific white shrimp *Litopenaeus vannamei*. *J. Crust. Biol.* 34 (3), 293–299.
- Ventura, T., Sagi, A., 2012. The insulin-like androgenic gland hormone in crustaceans: from a single gene silencing to a wide array of sexual manipulation-based biotechnologies. *Biotechnol. Adv.* 30, 1543–1550.
- Ventura, T., Manor, R., Aflalo, E.D., Weil, S., Rosen, O., Sagi, A., 2011a. Timing sexual differentiation: full functional sex reversal achieved through silencing of a single insulin-like gene in the prawn, *Macrobrachium rosenbergii*. *Biol. Reprod.* 86 (3):90. <http://dx.doi.org/10.1095/biolreprod.111.097261>.
- Ventura, T., Rosen, O., Sagi, A., 2011b. From the discovery of the crustacean androgenic gland to the insulin-like hormone in six decades. *Gen. Comp. Endocrinol.* 173, 381–388.
- Zhang, L., Yang, C., Zhang, Y., Li, L., Zhang, X., Zhang, Q., Xiang, J., 2007. A genetic linkage map of Pacific white shrimp (*Litopenaeus vannamei*): sex-linked microsatellite markers and high recombination rates. *Genetica* 131, 37–49.