Aquaculture 468 (2017) 471–480

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

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 $PL_{34–48}$. Grafting at PL_{37} 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.

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, Charniaux-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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http://dx.doi.org/10.1016/j.aquaculture.2016.11.007
0044-8486/

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 quelipeds 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). Aflalo 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_{12}) 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 pereiopods 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 $PL_{34–48}$ 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 = $PL_{34–48}$; 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_{37}; 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.