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Vitellogenin in hemolymph predicts gonad maturity in adult female *Litopenaeus (Penaeus) vannamei* shrimp

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ABSTRACT

Vitellogenin (Vtg), when measured in shrimp hemolymph has been found to reflect the degree of female shrimp ovarian development before eyestalk ablation, and to be a useful predictor of ovarian development after ablation. The present study evaluated correlations of vitellogenin in hemolymph with the number and diameter of oocytes in gonads, as a measure of reproductive status of Pacific white shrimp. Shrimps were grown at high density in a raceway, and evaluated at an average weight of 35 g. A significant Pearson correlation was found between Vtg and oocytes diameter (0.90 ± 0.08), but not for Vtg and number of oocytes, or for oocytes diameter and numbers. Vtg is the first reproductive trait found to predict reproductive capacity of Pacific white shrimp before eyestalk ablation, and shrimp producers can utilize it to define *a priory* which females to introduce into spawning tanks.

1. Introduction

Vitellogenesis is known as the process of biosynthesis of yolk proteins, and their transport and storage in the ovary (Charniaux-Cotton, 1985). Yolk proteins are accumulated in the egg, representing from 60% to 90% of total proteins (Quackenbush, 2001). The events that prepare the oocyte for yolk uptake, and the actual mechanics of yolk accumulation in penaeid shrimp has been fully described and divided in four to nine gonad stages based on histological analysis (Yano, 1988; Tan-Fermin and Pudadera, 1989; Mohamed and Diwan, 1994). Despite some differences on the nomenclature of oocyte types and maturation stages, these authors reported that during early or primary to late or secondary vitellogenesis, a considerable increase in the oocyte cell size is observed because of the yolk protein, vitellogenin (Vtg), accumulated in the cytoplasm (Meusy and Payen, 1988). For this reason, the frequency and diameter of oocytes have been utilized to compare differences in the ovarian maturation of penaeid females under different experimental conditions (Briarty, 1975; Yano, 1988; Tan-Fermin and Pudadera, 1989; Tan-Fermin, 1991; Mohamed and Diwan, 1994; Palacios et al., 1999).

Indeed, Vtg levels in penaeid shrimp hemolymph increase together with gonadosomatic index (Quackenbush, 1989; Wouters et al., 2001; Vazquez-Boucard et al., 2002) or in relation to specific qualitative gonadal stages evaluated histologically (Quinitio and Millamena, 1992; Quinitio et al., 1994; Jasmani et al., 2000; Tahara et al., 2005; Okumura et al., 2007). Recent works analyzed the relation between different indices of gonad development stages with special emphasis on quantitative traits such as oocyte diameter and other calculated indices (Palacios et al., 2003; Peixoto et al., 2003; Arcos et al., 2005a). Oocytes diameter measured histologically in a captive and ablated breeding population was the best reflection of the maturation condition of females in production (Palacios et al., 2003). Also oocytes diameter and a second trait, ovary maturity, derived from oocytes area and abundance, when evaluated in subadult families were significantly correlated to adult reproductive performance in the same families (Arcos et al., 2005a). However, none of those studies analyzed vitellogenin levels that represent a reliable quantitative trait that could be measured in a sample of hemolymph obtained from live animals, without the need to sacrifice them to obtain ovary samples for the evaluation of other traits. Recently, Ibarra et al. (2009) evaluated the heritabilities of two traits in unablated females, vitellogenin concentration in hemolymph and oocytes diameters, finding that these traits presented significant additive genetic variance to select from, and that they were highly genetically correlated. That study prompted the need to understand the basis of that large genetic correlation, such that vitellogenin in hemolymph could be proposed in a definitive manner as a predictor or indicator of gonad maturation stages if such an association existed.

The availability of an indicator of gonad maturation stage is important because it is known that during shrimp reproduction in captive conditions, only a fraction of the population spawns, and even a lower fraction is responsible for most nauplii production through multiple spawnings (for reviews see Racotta et al., 2003; Ibarra et al., 2007). Among several characteristics, multiple spawning females have the shortest latency to their first spawn (Palacios et al., 1999; Arcos et al., 2003a, 2004) suggesting a more advanced gonad development stage to achieve this first spawn. In a different work,

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Arcos et al. (2003b) found that Vtg concentration in hemolymph measured just before eyestalk ablation could be utilized as a possible predictor of maturation capacity induced by eyestalk ablation. Developing knowledge of predictors of reproductive traits measured before inducing reproduction is therefore important as it would allow nauplii producers at hatcheries to improve the output when utilizing only females in advanced maturation stages. Additionally, for selective breeding purposes, a trait that can be measured before the process of maturation is initiated in the laboratory would allow for designing a selective breeding program that utilizes multiple traits for selection of 'best' performers in one step at the end of the grow-out cycle. In a broader context, the evaluation of the relationship between concentration of Vtg in hemolymph and detailed ovarian development process through quantitative and qualitative traits obtained from histological analysis in a shrimp cultured population would be of considerable value for understanding the role of this lipoprotein dynamics on ovarian maturation. The present study aimed to evaluate the association between several qualitative and quantitative traits as gonad stages, oocytes numbers and diameters, and levels of hemolymph Vtg levels, with the goal of defining the most accurate and practical indicators of reproductive potential, of adult *Penaeus (Litopenaeus) vannamei* shrimp females.

2. Materials and methods

2.1. Animals

A captive population of *P. (Litopenaeus) vannamei* shrimp was used for the analyses (Ibarra and Famula, 2008). A total of 4080 juvenile (1–2 g) shrimp were stocked in a raceway at high density (400/m^2) at the commercial hatchery Acuacultores de La Peninsula de Baja California (APBC SA de CV, La Paz, Mexico), using a 308 m^2 rectangular raceway tank, provided with a continuous water flow (15 L/s). Supplemental aeration was used, maintaining oxygen levels greater than 4 mg/L. Feeding was done on demand, using a commercial pellet (40% protein) and tray-feeding. Food was added to the trays every 2 h. Density was periodically decreased by natural mortalities, and after 3 months in the raceway, density was adjusted to 50 shrimp per m^2 by randomly transferring out shrimp. At the end of a grow-out period of 10 months (June 2005–March 2006), the density in the raceway was 38 adult shrimp per m^2. At this time, male and female shrimp were placed in holding maturation tanks, fed with a diet containing 35% squid and 65% commercial pellet (40% protein), added in four daily rations after daily adjusting the amount to be equivalent to 20% live wet weight per shrimp.

2.2. Growth variables

After approximately 3 months, a total of 316 adult females with an average weight of 38.7 g were sampled, and length and weight of each female were measured and recorded. Relative condition index was determined using the ratio of wet body weight and length (Emmerson, 1980). Shrimp in good condition has a factor higher than 1.0 (high body weight/length ratio), in contrast to shrimp in poor condition with a factor lower than 1.0 (low body weight/length ratio).

$$W_t/aL^b = \text{Condition index} \approx 1$$

Where W_t is the shrimp weight and L is its length. Prior to using this equation, a lineal regression between individual weights and lengths was done to estimate both, a (intercept) and b (slope) of the regression line.

2.3. Vitellogenin concentration estimation

Three-hundred-microliter of hemolymph was obtained from the pleopod base of the first abdominal segment of each female, using a 1-mL syringe rinsed with cooled anticoagulant solution of 5% sodium oxalate in isotonic saline. The hemolymph was centrifuged at 1100 *g* for 2 min at 4 °C, and the resulting plasma was separated from the precipitated cells and stored at −80 °C for later analysis. For the analysis of vitellogenin (Vtg) concentration, vitellin (Vn) was purified from *P. vannamei* ovaries by high-performance liquid chromatography (HPLC), and polyclonal antibodies against *P. vannamei* (Vn) were prepared as described in Arcos et al. (2003a). The Vtg concentrations (μg/mL) were determined on the plasma with a quantitative enzyme-linked immunosorbent assay (ELISA). A standard curve was made with the purified Vn, and a linear regression was calculated to assess the relationship between optical density and the amount of purified Vn. All determinations for the calibration curve and tissue samples were done in duplicate. A number of quality criteria for validation of several steps of the assay were taken into account (data not shown) (Feldkamp and Smith, 1987; Mendoza et al., 1993).

2.4. Histological variables

After hemolymph sampling, the ovary from the entire cephalothoracic region up to the first abdominal segment was dissected and fixed in Davidson's solution for 24 h, embedded in a paraffin/paraplast mixture. Transverse sections (4 μm) of the ovary from the cephalothoracic region located at the approximate midpoint (anterior–posterior) of the hepatopancreas were obtained and stained with Harris haematoxylin-alcoholic eosin solution (Humanson, 1972). For quantitative histological analyses, each slide was examined under a microscope (10× and 40×) connected to a video camera (CoolSNAP-ProColor). Recorded images were digitalized using an image analysis program (Image Pro Plus version 4.5 software).

Each female was classified within one of nine developmental stages, defined by the presence of the most advanced oocyte type according to Tan-Fermin and Pudadera (1989), and Yano (1988): (a) chromatin nucleolus stage, (b) early perinucleolus stage, (c) late perinucleolus stage, (d) oil globule stage I, (e) oil globule stage II, (f) yolkless stage, (g) yolk granule stage, (h) prematuration stage, and (i) maturation stage. For each female, all oocyte types contained in the gonad were classified, counted, and measured. Classification of oocytes was done following Yano's, for each oocyte type: (1) chromatin nucleolus type, (2) early perinucleolus type, (3) late perinucleolus type, (4) oil globule stage I type, (5) oil globule stage II type, (6) yolkless type, (7) yolk granule type, (8) premature type, and (9) mature type. Four transects (vertical and horizontal) on each histological preparations were traced to obtain a square containing the complete surface of the gonad. For each female the following derived data was obtained: total number of oocytes, oocytes mean diameter (OD) which represents the weighted mean diameter (μm) of all oocytes in the ovary, ovary maturity (OM) representing the total area (μm^2) occupied by the oocytes present in each female gonad, and total ovary area which represents the complete surface area (μm^2) occupied by the gonad (Table 1) (Arcos et al., 2005a).

2.5. Statistical analysis

Pearson's correlations were obtained to define any association between the studied variables, including female's total weight and condition index.

All females in the population were grouped into the nine gonad developmental stages as previously described. Analysis of variance was undertaken to establish if total weight (g), condition index, vitellogenin (μg/mL), number of oocytes, oocytes mean diameter (μm), ovary maturity (μm^2), and total ovary area (μm^2), depended on