Effects of Dietary Sterols on Growth, Survival, and Midgut Gland Histology in Juvenile Prawns, *Artemesia longinaris* (Decapoda, Penaeidea)

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Abstract

Two trials were conducted to evaluate the growth, survival, and midgut gland histology of Argentine prawn, *Artemesia longinaris*, nourished with diets containing different sterols. Juveniles of 0.97-1.01 or 1.75 g were fed one of four semi-purified diets containing 2% cholesterol, ergosterol, stigmasterol, or β-sitosterol, or a control diet containing no sterols for six weeks. The digestive glands of intermolt prawns were dissected out and processed for light microscope study. Diets containing cholesterol or stigmasterol resulted in alterations such as the dismissing of cellular height, loss of the star-shaped lumen of the tubules, retraction of basal membranes and absence of the brush border, hemocytic infiltration, cells with foamy appearance, cellular necrosis and hypertrophy, and tissue disorganization. Results suggest that adding cholesterol or stigmasterol to feeds for this species promotes weight increase and a hepatopancreas with a histological structure typical to that of wild prawns.

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Introduction

The commercially valuable prawn, Artemesia longinaris, is distributed along the coastal waters of Argentina, Uruguay, and Brazil from 22°30'S to 43°20'S (Boschi, 1963). The greatest abundance of this species occurs in Mar del Plata and Rawson, Argentina. The annual catches of A. onginaris are highly variable and, therefore, it is important to determine the feasibility of culturing it on a commercial scale.

One of the main problems of growing penaeid shrimps is inadequate knowledge of their nutritional requirements. While nutritional principles are similar for all animals, the levels of required nutrients in feeds varies with species. Dietary sterols are needed for growth and survival in prawns (Petriella et al., 1984). Sterols are essential for crustaceans, which are unable to synthesize these compounds. Important components such as molting hormones, sex hormones, bile acids, and vitamin D are synthesized from cholesterol (Teshima and Kanazawa, 1971) Cholesterol is a membrane component involved in the absorption and transport of fatty acids (Akiyama et al., 1991) and is obtained directly from the diet or via the metabolic conversion of other dietary sterols (Kanazawa, 2001). Some species of Crustacea possess the ability to dealkylate some C<sub>28</sub> and C<sub>29</sub> sterols such as sitosterol, ergosterol, and stigmasterol via desmosterol to cholesterol (Kanazawa et al., 1971; Teshima and Kanazawa, 1986).

The midgut gland, also known as the digestive gland or hepatopancreas, is the largest organ by volume in decapod crustaceans and has many biological functions including synthesis and secretion of digestive enzymes, absorption of digested products, maintenance of mineral reserves and organic substances, lipid and carbohydrate metabolism, distribution of stored reserves during the molt cycle, and catabolism of some organic compounds (Ceccaldi, 1997). There is a close correlation between the nutritional state of an animal and the histology of its midgut gland (Vogt et al., 1985; Díaz et al., 2006). The midgut gland is essentially composed of tubes lined by a simple epithelium with four cell types: E (embryonic), F (fibrillar), R (resorptive), and B (blisterlike) that are surrounded by connective tissue (Petriella and Fonalleras, 1998). E cells are situated at the ends of tubules and develop into R cells that are involved in absorption, catabolism, storage of nutrients, and detoxification of heavy metals. F cells synthesize digestive enzymes (Icely and Nott, 1992). B cells have absorptive and degradative functions (Vogt, 1994; Johnston et al., 1998). A bilobed brown organ occupies much of the cephalothoracic cavity in A. longinaris and is connected to the pyloric stomach by two ducts (Petriella and Fonalleras, 1998). The organ is enclosed in a laminar connective tissue capsule and the morphological unit consists of a blind ending tubule with the four types of cells, as in other crustaceans.

The aim of this work was to study the effects of dietary sterols on growth, survival, and histological changes of hepatopancreatic cells in A. longinaris juveniles under culture conditions.

Materials and Methods

Two experiments of six weeks each were conducted. Prawns were obtained from a commercial fisherman in the coastal waters of Mar del Plata, Argentina (38°S), and maintained in fifteen 150-l glass aquaria with marine water and filter beds of shell and sand.

Five isoproteic semi-purified diets (44.5% protein, 8.7% lipids, 5.9% ash, 7.1% moisture) were prepared by the cold extrusion method (Fenucci, 1981). The control diet contained no sterols; the experimental diets contained 2% cholesterol, ergosterol, β-sitosterol, or stigmasterol, added to the basal control diet (Table 1).

Groups of 18 prawns/m² (0.97-1.01 g in experiment 1; 1.75 g in experiment 2) were stocked in triplicate aquaria. Prawns were acclimated for seven days and fed the basal diet *ad libitum* daily. During the feeding trials, the amount of food was adjusted according to their requirements. Uneaten food, dead prawns, and exuviae were removed daily before feeding. Two additional groups of animals were maintained in total starvation to evaluate the histology of the midgut gland in this severe condition.
Table 1. Composition of the basal diet for Argentine prawns (g/100 g diet).

<table>
<thead>
<tr>
<th>Sterols</th>
<th>Minerals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>Calcium 1000 mg; magnesium 500 mg; potassium 99 mg; zinc 30 mg; iron 10 mg; copper 2 mg; iodine 150 µg; selenium 200 µg; molybdenum 500 µg (Twin Laboratories, Inc., USA)</td>
</tr>
<tr>
<td>Corn starch</td>
<td>Calcium pantothenate 35; menadione 34; vitamin A acetate 100; calcium pantothenate 250; choline chloride 300; vitamin E (Laboquímica Argentina) 1500</td>
</tr>
<tr>
<td>Gelatin</td>
<td>phosphorus 99 mg; riboflavin 156; pyridoxine hydrochloride 156; niacin 150; inositol 300; ascorbic acid 400; biotin 32; folic acid 25; niacin 150; pyridoxine 213; vitamin K 20; vitamin B12 20; vitamin B6 20</td>
</tr>
<tr>
<td>Cellulose</td>
<td>Vitamins 3: vitamin B1 2.0; vitamin B2 2.0; vitamin B6 2.0; vitamin B12 2.0; vitamin K 2.0; vitamin C 2.0; vitamin D 2.0; vitamin E 2.0; vitamin A 2.0; vitamin B12 2.0; vitamin B6 2.0; vitamin C 2.0; vitamin D 2.0; vitamin E 2.0; vitamin A 2.0; vitamin B12 2.0; vitamin B6 2.0; vitamin C 2.0; vitamin D 2.0; vitamin E 2.0; vitamin A 2.0</td>
</tr>
<tr>
<td>PUFA1</td>
<td>Glutamic acid 200; L-carnitine hydrochloride 200; DL-α-tocopherol 200; thymol 200; carvacrol 200; methyl salicylate 200; propionic acid 200; L-lysine hydrochloride 200; L-cysteine hydrochloride 200; sodium alginate 200; sodium chloride 200; sodium citrate 200; sodium nitrate 200; sodium bicarbonate 200; sodium dihydrogen phosphate 200; sodium hydroxide 200; sodium hydrogen carbonate 200; sodium chloride 200; sodium citrate 200; sodium nitrate 200; sodium bicarbonate 200; sodium dihydrogen phosphate 200; sodium hydroxide 200; sodium hydrogen carbonate 200; sodium chloride 200</td>
</tr>
<tr>
<td>Sodium alginate</td>
<td>Sterols 1: cholesterol 1.0; stigmasterol 1.0; β sitosterol 1.0; ergosterol 1.0</td>
</tr>
<tr>
<td>Squid protein2</td>
<td>Sterols 2: cholesterol 1.0; stigmasterol 1.0; β sitosterol 1.0; ergosterol 1.0</td>
</tr>
<tr>
<td>Vitamin mix2</td>
<td>Sterols 3: cholesterol 1.0; stigmasterol 1.0; β sitosterol 1.0; ergosterol 1.0</td>
</tr>
<tr>
<td>Hexametafosfate</td>
<td>Sterols 4: cholesterol 1.0; stigmasterol 1.0; β sitosterol 1.0; ergosterol 1.0</td>
</tr>
<tr>
<td>Soy lecithin</td>
<td>Sterols 5: cholesterol 1.0; stigmasterol 1.0; β sitosterol 1.0; ergosterol 1.0</td>
</tr>
<tr>
<td>Minerals 4</td>
<td>Sterols 6: cholesterol 1.0; stigmasterol 1.0; β sitosterol 1.0; ergosterol 1.0</td>
</tr>
</tbody>
</table>

1 Polyunsaturated fatty acids (Omega Sur, Mar del Plata, Argentina)
2 Diaz et al. (1999)
3 mg/kg: thiamin 163; riboflavin 156; pyridoxine 213; cianocobalamín 20; biotin 250; folic acid 25; niacin 250; inositol 300; ascorbic acid Rovimix STAY C (Sigma) 781; cholecaliferol 35; menadione 34; vitamin A acetate 100; calcium pantothenate 250; choline chloride 300; vitamin E (Laboquímica Argentina) 1500
4 Calcium 1000 mg; magnesium 500 mg; potassium 99 mg; zinc 30 mg; iron 10 mg; copper 2 mg; iodine 150 µg; selenium 200 µg; molybdenum 500 µg (Twin Laboratories, Inc., USA)

Water temperature was 20±2°C, salinity 33‰, and pH 7. Prawns were weighed at the beginning and end of the experiments (44 days). At the end of the trials, individuals in the intermolt stage were selected for histological study: midgut glands were removed and fixed for 24 h in Davidson’s fluid (Bell and Lightner, 1988), dehydrated in increasing concentrations of ethanol, clarified with benzene, and embedded in paraffin. Tissue sections of 3 µm were stained with hematoxylin-eosin, examined by light microscopy, and photographed. Molt stage was determined by examining the uropod setae development (Petriella, 1984).

Data were analyzed using Bartlett’s test to determine the homoscedasticity of variances. One-way analysis of variance (ANOVA) was used to determine significant differences between treatments and the chi-square test for survival (Sokal and Rohlf, 1995). Significance was set at p<0.05.

Results

At the end of both experiments, final mean weight and percent weight gain of prawns fed the diets containing cholesterol or stigmasterol were statistically higher than those of prawns fed the control (Table 2). The final mean weight and percent weight gain of individuals fed the diet containing ergosterol were not included in the comparison because survival in this treatment was very low.

Tubules through the middle region of animals fed the cholesterol or stigmasterol diets exhibited a cell structure and star-shaped lumina similar of those of wild prawns (Fig. 1). There was a marked brush border on the luminal surface of the cells, a myo-epithelial layer surrounding the tubules, and hemocytes were scarce and confined to hemal spaces between the tubules. The midgut gland of individuals fed the β-sitosterol diet showed hemocyte infiltration, cellular retraction, desquamation, and wavy basal membranes and many tubular lumina lost their star-like form and brush border (Fig. 2). In the midgut glands of prawns fed the ergosterol diet, tubules were separated due to cellular retraction, vacuolization was more prominent, and the cells had a foamy appearance. Individuals fed the sterol-free diet evidenced hypertrophy and shrinkage of cells, and ample tubular lumina; some areas revealed hemocytic encapsulation and severe necrotic focus (Fig. 3). After 14 days starvation, cell vacuolization was more prominent and certain parts of the midgut glands of some animals lost the typical acinar structure of the hepatopancreatic tubules and disorganized tissues (Fig. 4).

Table 2. Mean weight, weight gain, and survival of Artemesia longinaris fed diets containing cholesterol, stigmasterol, β sitosterol, ergosterol, or the sterol-free control diet.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Mean wt (g)</th>
<th>Wt gain (%)</th>
<th>Survival (%)</th>
<th>Mean wt (g)</th>
<th>Wt gain (%)</th>
<th>Survival (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.00±0.195</td>
<td>1.55±0.292</td>
<td>55.07±2</td>
<td>1.75±0.314</td>
<td>2.74±0.227</td>
<td>56.95±4</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>1.01±0.223</td>
<td>1.89±0.415</td>
<td>69.66±5</td>
<td>3.48±0.561</td>
<td>99.29±1</td>
<td>74.00±3</td>
</tr>
<tr>
<td>Stigmasterol</td>
<td>1.00±0.203</td>
<td>1.87±0.375</td>
<td>86.50±7</td>
<td>3.42±0.622</td>
<td>96.06±1</td>
<td>74.00±3</td>
</tr>
<tr>
<td>β sitosterol</td>
<td>0.97±0.214</td>
<td>1.65±0.409</td>
<td>69.72±5</td>
<td>3.05±0.528</td>
<td>74.77±5</td>
<td>74.00±3</td>
</tr>
<tr>
<td>Ergosterol</td>
<td>0.99±0.207</td>
<td>-</td>
<td>16.66±5</td>
<td>1.75±0.367</td>
<td>-</td>
<td>29.63±5</td>
</tr>
</tbody>
</table>

Different superscripts in a column indicate significant differences (p<0.05).
Fig. 1. Transverse sections of tubules through the middle regions of prawns (*Artemesia longinaris*) fed diets containing (a) stigmasterol (bb = brush border, E = embryonic cells, F = fibrillar cells, R = resorptive cells) or (b) cholesterol (hs = hemal sinus, L = lumen, ml = myo-epithelial layer). Bar = 25 μ.

Fig. 2. Transverse sections of tubules through the middle regions of prawns (*Artemesia longinaris*) fed diets containing (a) β-sitosterol (bl = wavy basal membranes, h = hemocytes) or (b) ergosterol (ts = tubular separation, V = foamy cells with vacuoles). Bar = 25 μ.

Fig. 3. Transverse sections of tubules through the middle regions of prawns (*Artemesia longinaris*) fed a sterol-free diet had (a) B = hypertrophy of B cells, tl = large tubular lumina, and (b) nf = a necrotic focus. Bar = 25 μ.
the epithelia where cholesterol is absorbed (Martinez Romero et al., 1991).

Cholesterol accounts for 90-95% of the sterol composition in crustaceans (Kanazawa, 2001). After 30 days, the total sterol content in the body tissues of *Marsupenaeus japonicus* dropped in prawns fed a sterol-free diet but, in individuals fed diets containing ergosterol, stigmasterol, or β-sitosterol, the sterol content was similar to that of the prawns before the feeding trial (Kanazawa et al., 1971), suggesting that cholesterol and other sterols are absorbed and utilized. Since the percent cholesterol was high and there were no detectable increases of other sterols, it may be that *M. japonicus* convert C_{28} and C_{29} sterols to cholesterol (Kanazawa et al., 1971). In both our 6-week experiments, the diets containing cholesterol, stigmasterol, or β-sitosterol sustained growth.

The bioconversion rates and consequent nutritional values of sterols other than cholesterol in Crustacea may be species specific (D’Abramo et al., 1984). The effective utilization of some dietary phytosterols apparently reflects the feeding habits of omnivorous species compared with the apparently exclusive requirement for cholesterol of carnivorous crustaceans (D’Abramo and Daniels, 1994). Total replacement of cholesterol with a mixture of phytosterols composed primarily of β-sitosterol did not yield good growth and survival in juvenile lobsters, *Homarus sp.* (D’Abramo et al., 1984). On the other hand, a mixture of phytosterols is as efficient as cholesterol in satisfying the dietary sterol requirement of juvenile *Macrobrachium rosenbergii* (D’Abramo and Daniels, 1994). The present study shows that cholesterol and stigmasterol diets have beneficial effects on growth but do not affect survival in the omnivorous *A. longinaris*.

Changes in the histology of the hepatopancreas can be observed before other body responses (Vogt et al., 1985; Esteve and Herrera, 2000). The midgut gland cytology of *A. longinaris* maintained at 16‰ salinity revealed epithelial necrosis, hemocytic nodules, and epithelial desquamation due to changes in environmental conditions (Masson, 2001). Tissues from the midgut gland of crustaceans seem to be very sensitive to feed ingredients. There were differences in the structure of the midgut gland of *Palaemonetes argentinus* provided diets with different levels of cholesterol (Diaz et al., 2002) and of *Penaeus monodon* given antinutritional factors present in grain legumes (Kumaraguru Vasagam et al., 2007). Variations in the cytological characteristics of the midgut gland of *A. longinaris* were induced by suboptimal levels of dietary vitamin E and A (Fernández Gimenez, 2002). Histological alterations were marked in cells of young *A. longinaris* fed diets deficient in methionine or containing levels of methionine higher than 10%; the most notable changes being shrinkage of cells and microvilli, condensation of nuclear chromatin, cellular hypertrophy, cellular desquamation towards the tubular lumen, hemocytic infiltration, nodules or tumor structures, and general collapse of tubules and cellular necrosis (Romanos Mangialardo, 2006). In our study, *A. longinaris* fed diets with

**Discussion**

Most animals are capable of synthesizing sterols from acetate, but not crustaceans (Teshima and Kanazawa, 1971). Crustaceans need an external source of sterols (Martinez Romero et al., 1991; Haran and Fenucci, 1996, 2008). Cholesterol may be a constituent of membranes and a precursor of steroid hormones, molting hormones, and cholesteryl esters in penaeid shrimps. The required level of dietary cholesterol for invertebrates ranges 0.1-2.0% of the dry weight of diets (Kanazawa, 2001). Whereas 0.5-2.0% is optimal for survival in *A. longinaris*, the optimum level for digestibility of dietary cholesterol is 2.3%, suggesting the probable existence of a saturable carrier system at the level of the epithelium of the tubules where cholesterol is absorbed.
β-sitosterol or ergosterol showed alterations, infiltration of hemocytes, cellular retraction, and cells with foamy appearance. In individuals fed the sterol-free diet, focus of encapsulation, vacuolization and cellular retraction, and desquamation were observed. In animals starved for 14 days, the hepatopancreas showed tubular disruption.

In conclusion, under the experimental conditions, diets containing cholesterol or stigmasterol showed good results for growth of prawn and the histology of the hepatopancreas of prawns fed these diets was similar to that of wild individuals.

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References
Sterols in diets of Artemesia longinaris


