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Research Article

Effect of salinity changes on the midgut gland of *Artemesia longinaris* (Decapoda, Penaeidae)

Ignacio Masson[1, 3], Ana C. Díaz[1, 3] & Ana M. Petriella[2, 3]
[1]Comisión de Investigaciones Científicas de la Provincia de Buenos Aires, Argentina
[2]Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina
[3]Departamento de Ciencias Marinas, Universidad Nacional de Mar del Plata, Argentina
Funes 3350, B7602AYL, Mar del Plata, Argentina

ABSTRACT. The response of the midgut gland of *Artemesia longinaris* to salinity changes was evaluated by analyzing its histological changes. Animals were exposed gradually and abruptly to 33, 29, 25 and 16 psu for different time intervals and readapted to 33 psu for 30 days. Individuals maintained 10 days at 16 psu showed the lowest survival and presented histopathologies which were not present in those readapted to 33 psu. Shrimps abruptly transferred from 33 to 16 psu died in 3-5 h but did not show midgut gland alterations likely due to the brief exposure. Only shrimps abruptly transferred from 33 to 25 psu presented histopathologies after 96 h. When readapted to 33 psu for 30 days, the midgut gland recovered an unaltered structure. Except E-cells, which did not vary in height among treatments, F, R and B-cells were taller in animals gradually adapted to 29 than to 16 psu. Abrupt salinity changes had a significant effect on the mean height of F, R and B-cells of those animals transferred from 33 to 25 psu (from 24 to 96 h after transfer F and R-cells heights decreased, and from 96 to 144 h after transfer B-cells height increased). Our study shows the effect of osmotic stress at the tissue level on the midgut gland and, at least partially, explains the reason for the mortalities at low salinities.

Keywords: midgut gland, hepatopancreas, osmotic stress, histopathology, salinity, *A. longinaris*, Argentina.

Efecto de los cambios de salinidad sobre el hepatopáncreas de *Artemesia longinaris* (Decapoda, Penaeidae)

RESUMEN. Se evaluó la respuesta del hepatopáncreas de *Artemesia longinaris* a los cambios de salinidad. Los animales fueron expuestos gradual y abruptamente a 33, 29, 25 y 16 psu durante distintos intervalos y readaptados a 33 psu por 30 días. Los individuos gradualmente adaptados a 16 psu tuvieron baja supervivencia y presentaron alteraciones histológicas ausentes en los readaptados a 33 psu. Aquellos transferidos repentinamente de 33 a 16 psu murieron en 3-5 h pero no mostraron alteraciones, posiblemente debido a la breve exposición. Sólo los transferidos repentinamente de 33 a 25 psu por más de 96 h presentaron daño tisular. Cuando se readaptaron a 33 psu durante 30 días, el hepatopáncreas mostró una estructura normal. Exceptuando las células E que no variaron en altura entre tratamientos, las F, R y B fueron más altas en los animales gradualmente adaptados de 33 a 29 psu. Los cambios súbitos de salinidad tuvieron un efecto significativo en la altura media de las células F, R y B solamente en animales transferidos de 33 a 25 psu (24 a 96 h después de la transferencia disminuyó la altura de las células F y R, y 96 a 144 h después hubo un aumento en la altura de las células B). El presente estudio muestra el efecto de los cambios de salinidad sobre el hepatopáncreas y explica, al menos parcialmente, la razón de la mortalidad a bajas salinidades.

Palabras clave: glándula digestiva, hepatopáncreas, estrés osmótico, alteraciones histológicas, salinidad, *A. longinaris*, Argentina.

Corresponding author: Ignacio Masson (ignaciomasson@yahoo.com)

INTRODUCTION

Artemesia longinaris is a commercially important marine shrimp that inhabits coastal waters of Argentina, Uruguay, and South Brazil, where temperatures range from 8 to 22ºC and salinities between 33 and 36 psu (Boschi, 1969; Boschi & Scelzo, 1974; Boschi & Gavio, 2005). Unlike many

other species of penaeid shrimps that can tolerate a wide range of salinities, such as *Penaeus vannamei, P. setiferus* (Briggs *et al*., 2004) and *P. aztecus* (Venkataramiah *et al.*, 1974; Saoud & Davis, 2003), *A. longinaris* is strictly marine during all phases of its life cycle (D'Incao, 1999).

There are many publications covering the growth and survival of a variety of penaeid species at different salinity levels and suggesting the optimal salinity range for culture (Aziz & Greenwood, 1981; Ogle *et al.*, 1992; Brito *et al.*, 2000; Saoud & Davis, 2003; Buckle *et al.*, 2006). However, the actual causes of impairment in growth and survival as a result of adverse salinity levels are unknown.

The midgut gland is the primary organ responsible for digestion and storage of ingested materials in crustaceans (Factor, 1995). It has been noted that this organ undergoes histological and histochemical modifications in response to physiological demands, such as molt (Al-Mohanna & Nott, 1989) and reproduction (Díaz *et al*., 2006), osmotic variations (Díaz *et al*., 2010) pollution (Popescu-Marinescu *et al.*, 1997), and that there is a correlation between the midgut gland structure and the animal's physiological condition and nutrition (Piedad-Pascual *et al*., 1983; Fernández-Giménez *et al.*, 2008; Fenucci *et al*., 2009).

The midgut gland of wild *A. longinaris* was described by Petriella & Fonalleras (1997). Like in other decapods, it is composed of numerous blind-ended tubules that communicate with the midgut. These tubules are lined by a simple, columnar epithelium composed of E, F, R and B-cells (Díaz *et al.*, 2006). The E-cells or embrionary cells are undifferentiated cubic cells, with a nucleus that occupies most of the cytoplasm (Icely & Nott, 1992). They are located in the distal zone of blind-ended tubules and as they divide they originate the other cell types (Johnston *et al.*, 1998). The F-cells or fibrillar cells are cylindrical cells, with a central nucleus characterized by high activity producing digestive enzymes (Loizzi, 1971; Rangneker & Momin, 1974; Vogt, 1994). The R-cells or resorptive cells are the most common cell type. The nucleus is basal and the cytoplasm includes a large number of vacuoles which are products of lipid and glycogen storage and, to a lesser extent, copper and other metals. They perform digestion by contact in the enteric surface followed by molecular transport through their brush border (Loizzi, 1971; Al-Mohana & Nott, 1989; Icely & Nott, 1992). The blister-like cells (B-cells) present a large vacuole that occupies most of their cytoplasm. They carry out intracellular digestion, absorbing the remaining products of digestion and excreting waste products (Bunt, 1968; Loizzi, 1971; Icely & Nott, 1992).

Because the function of the midgut gland determines, along with other vital organs, the growth and survival of crustaceans, was studied its response to variations in the salinity of the media by noting histopathological changes and by observing the changes in height of the cell types within the midgut gland. In addition, it was studied whether there is a difference in the type of response of the midgut gland to gradual compared to abrupt salinity changes, and the recovery capacity of the organ when animals were readapted to their optimal salinity range.

MATERIALS AND METHODS

Adult shrimps (1.5-3.0 g) at sexual rest were captured in the coastal waters of Mar del Plata (37º56'S, 57º45'W). They were brought to the lab and distributed in 150 L aquaria with 33 psu natural seawater at a density of 20 ind aquaria. Water temperature was 20 ± 2°C and the photoperiod used was 10 h light: 14 h dark. Aeration was supplied continuously by air stones connected to a regenerative blower. Aquaria had sand and shell filter beds. Shrimps were fed once daily with an artificial pelletized diet prepared by the cold extrusion method (45% protein, 7% lipid, 7% moisture, 7% ash) (Fenucci *et al*., 1981). Exuviae, dead animals, and uneaten food were removed to preserve water quality.

This study was divided into two separate trials that differed in how quickly the salinity was changed: 2-3 psu day^{-1} (gradual) *versus* abrupt change. In both trials, the midgut glands were processed in the same manner. Cephalothorax were fixed in Davidson's solution (ethanol, acetic acid, formalin, and distilled water) for 24 h (Bell & Lightner, 1988). After fixation, they were cut sagittally into two halves, dehydrated in increasing concentrations of ethanol, butyl alcohol (two changes of 24 h), butyl-paraffin 50:50 (48 h), and finally embedded in paraffin. Three-µm sections were cut with a microtome and stained with hematoxylin-eosin (H&E) and periodic acid-Schiff (PAS). Sections were examined using an Olympus photomicroscope furnished with an ocular micrometer. Measurements of the height of 10 cells randomly selected per cell type were taken at the medial and proximal zones of the tubules, except for E-cells which were measured at the distal zone, the only zone in which they are present. Cell height values are presented as mean ± standard error. Photo-micrographs of the slides were taken. Salinity measurements were performed using a hand refrac-tometer.

First trial: gradual salinity changes

After maintaining the shrimps for a week at 33 psu in eight separate aquaria, salinities were gradually changed to 29, 25 and 16 psu with two aquaria per salinity treatment and 20 animals aquarium. Two aquaria were maintained at 33 psu until the end of the trial as a control. Salinities were adjusted by diluting the media with freshwater (0 psu, previously aged tap water) at a rate of 2-3 psu day^{-1}. After 30 days, except for the animals held at 16 psu which were maintained only 10 days at this salinity because of the high mortality, 50% of the individuals from each treatment were randomly removed, fixed and examined for histopathological changes in the midgut gland. The remaining animals were gradually readapted to 33 psu and after 30 days they were fixed and examined for histopathological changes in the midgut gland. These animals readapted from 16, 25, 29 and 33 psu back to 33 psu for 30 days will henceforth be referred to as recovered from 16, 25, 29 and 33 (no change, control) psu, respectively. Survival rates were calculated at 10, 20, 30, 40, 50 and 60 days from the start of the experiment.

Second trial: abrupt salinity changes

Shrimps maintained at 33 psu during one week were abruptly transferred to 16, 25 and 29 psu at a density of 20 ind aquaria, and two aquaria per salinity treatment. Two aquaria with 20 ind each were maintained at 33 psu as control treatments. After 24, 48, 96 and 144 h, 10 ind from each aquarium were randomly removed, fixed and examined for histopathological changes in the midgut gland.

Statistical analysis

The survival rates were calculated by taking into account the number of dead animals within 10, 20, 30, 40, 50 and 60 days, where applicable, for each salinity treatment. Significant differences in survival among treatment were tested using the Chi-square test (alpha: 0.05). One-way ANOVAs (with Tukey's HSD *Post-hoc* tests) were used to test for significant differences in mean cell height among treatments for both trials (alpha: 0.05). Only cells belonging to the same type were considered in each test, with the grouping factor being the salinity treatment. SPSS 11.5 (SPSS 2002) was used both for the survival analysis and the cell-height comparisons.

RESULTS

First trial: gradual salinity changes

Survival rates were not significantly different among salinities of 25, 29 and 33 psu (70, 68 and 70%, respectively). However, animals held at 16 psu had a ten-day survival of 43%, which was significantly lower than the survival at any of the other salinities ($P < 0.05$) (Fig. 1). Because of the low survival at 16 psu, this treatment was discontinued on day 10 by gradually reverting the salinity to 33 psu.

The midgut glands of the shrimps held at 25, 29 and 33 psu presented a normal histological structure (Fig. 2). In contrast, the midgut glands of the shrimps maintained at 16 psu for 10 days showed histological changes: necrotic foci, atrophy of the tubules, and infiltration of hemocytes, which were sometimes forming nodules, encapsulating injured tubules and cellular debris. There was neither granular content nor PAS-positive material in the lumen of the tubules, in contrast to the other treatments (Fig. 3). Another pathological characteristic was the presence of epithelial sloughing (*i.e.*, cell exfoliation) into the tubular lumen (Fig. 4).

Animals held at 16 psu for 10 days and readapted to 33 psu for 30 days did not show any pathological signs in the midgut gland. They presented a structure comparable to that of the control animals maintained at 33 psu.

There were no significant differences in mean height of E-cells among treatments. Contrarily, F-cells attained greater heights in those animals maintained 30 days at 29 psu (38.2 ± 2.42 µm) than in those maintained 10 days at 16 psu (27.8 ± 0.81 µm) ($P < 0.05$). Only those animals readapted from 29 to 33 psu showed a significant change in F-cell mean height value, which decreased from 38.2 ± 2.42 µm at 29 psu to 29.7 ± 1.45 µm at 33 psu ($P < 0.05$) (Fig. 5a). There were no statistical differences in F-cell mean height values among the remaining treatments.

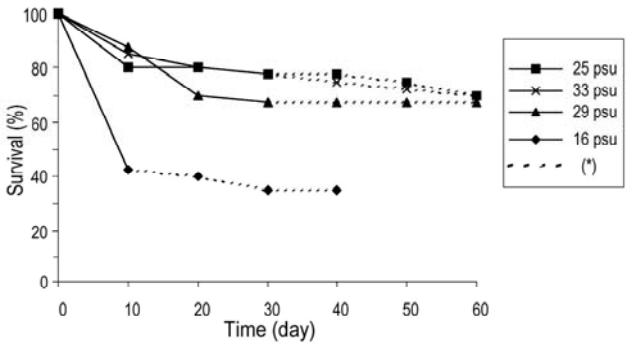

Figure 1. Survival (%) of *A. longinaris* exposed to gradual salinity changes. (*) Salinity gradually reverted to 33 psu.

Figura 1. Supervivencia (%) de *A. longinaris* expuesto a cambios graduales de salinidad. (*) Salinidad gradualmente revertida a 33 psu.

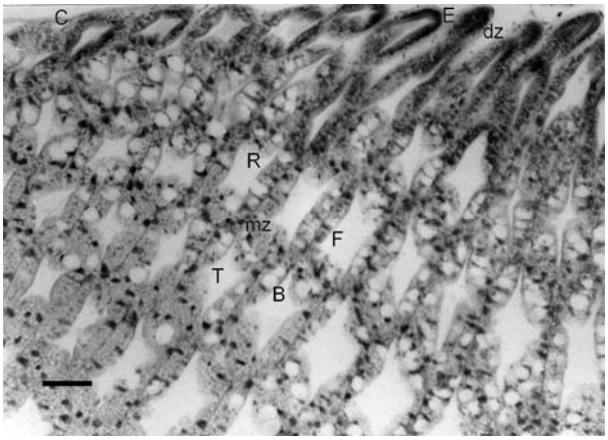

Figure 2. View of the distal and medial zones of the midgut gland tubules of *A. longinaris* maintained at 29 psu for 30 days; normal structure; H&E. B: B-cell, C: connective tissue capsule, dz: distal zone, E: E-cell, F: F-cell, mz: medial zone, R: R-cell, T: tubule. Scale bar: 100 µm.

Figura 2. Vista de la zona distal y media de los túbulos del hepatopáncreas *A. longinaris* mantenidos a 29 psu durante 30 días; estructura normal; H&E. B: célula B, C: cápsula de tejido conectivo, dz: zona distal, E: célula E, F: célula F, mz: zona media, R: célula R, T: túbulo. Escala: 100 µm.

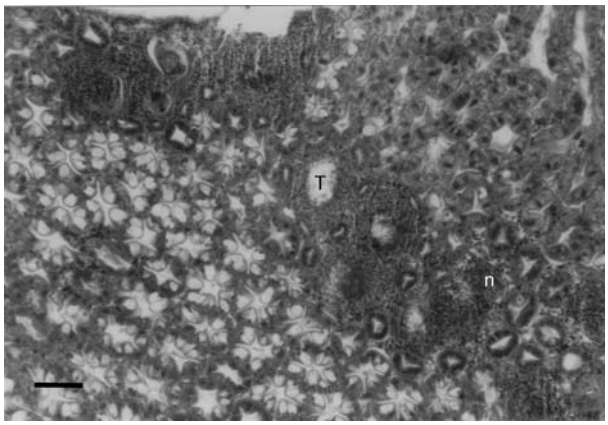

Figure 3. View of the midgut gland of *A. longinaris* maintained 10 days at 16 psu showing many hemocytic nodules and necrotic tubules; PAS. n: nodule, T: tubule. Scale bar: 100 µm.

Figura 3. Vista del hepatopáncreas de *A. longinaris* mantenidos 10 días a 16 psu mostrando nódulos hemocíticos y túbulos necróticos; PAS. n: nódulo, T: túbulo. Escala: 100 µm..

R-cells were significantly higher in those animals maintained at 29 psu (40.1 ± 2.13 µm) than in those animals in the remaining treatments ($P < 0.05$), except

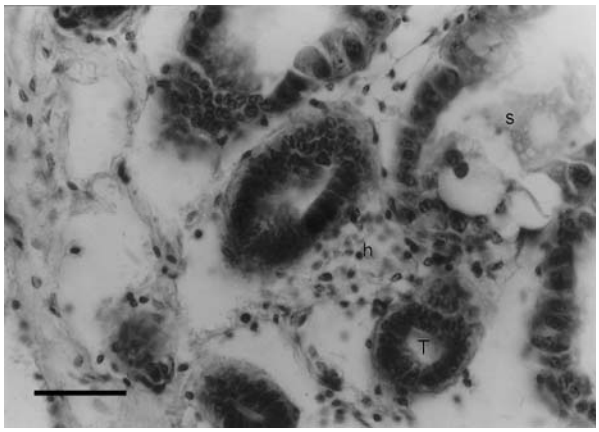

Figure 4. Infiltration of hemocytes into the intertubular space of the midgut gland of *A. longinaris* maintained 10 days at 16 psu. Note the thickened intertubular space occupied by connective tissue and hemocytes; H&E. h: hemocytes, s: sloughing of tubule epithelium, T: tubule. Scale bar: 50 µm.

Figura 4. Infiltración de hemocitos en el espacio intertubular del hepatopáncreas de *A. longinaris* mantenidos 10 días a 16 psu. Notar el espacio intertubular engrosado, ocupado por tejido conectivo y hemocitos; H&E. h: hemocitos, s: desprendimiento del epitelio tubular, T: túbulo. Escala: 50 µm.

for those maintained 30 days at 33 psu (35.8 ± 2.57 µm) and those recovered from 25 psu (35.6 ± 1.06 µm). Animals maintained at 16 and at 25 psu, recovered from 29 psu, and those maintained 60 days at 33 psu showed no statistical differences in R-cell mean-height values (Fig. 5b).

B-cells were taller in those animals recovered from 25 psu (45.4 ± 1.68 µm) ($P < 0.05$). This mean height value was not statistically different from the one for animals maintained at 29 psu (42.5 ± 3.41 µm). In addition, no significant differences in mean height values were found among the latter treatment and the treatments including the animals recovered from 16 psu (39.6 ± 1.65 µm), those maintained at 25 psu (38.8 ± 1.75 µm), those recovered from 29 psu (39.6 ± 1.14 µm), and those maintained 60 days at 33 psu (35.8 ± 1.58 µm) (Fig. 5c).

Coincidentally for F, R and B cell-types, the mean heights attained at 16 psu were in all cases significantly lower than the mean-heights attained at 29 psu ($P < 0.05$).

Second trial: abrupt salinity changes

Animals abruptly transferred from 33 to 16 psu developed a whitish coloration in their pleons and died

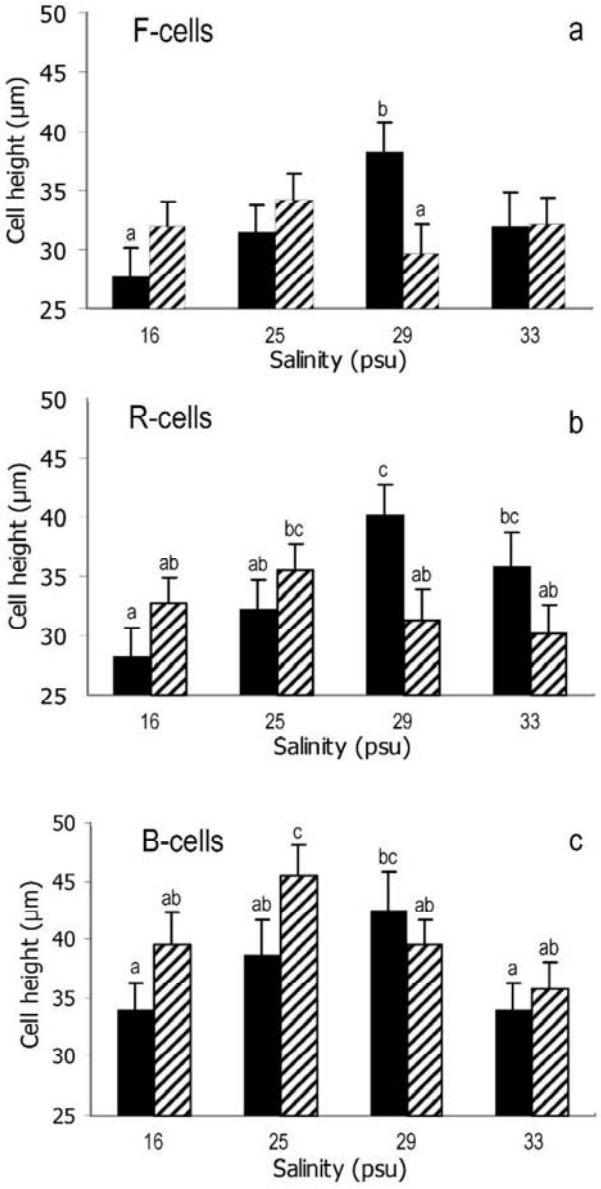

Figure 5. Mean heights (µm) of F (a), R (b) and B-cells (c) of *A. longinaris* maintained at different salinities for 30 days* (solid bars) and then gradually readapted to 33 psu (hatched bars). Error bars represent the positive standard error. Different letters indicate statistical differences in mean values among treatments for a same cell type ($P < 0.05$). (*) The time of exposure to 16 psu was for 10 days only due to the low survival.

Figura 5. Altura media (µm) de las células F (a), R (b) y B (c) de *A. longinaris* mantenidos a distintas salinidades durante 30 días* (barras sólidas) y gradualmente, readaptados a 33 psu (barras sombreadas). Las barras de error representan el error estándar positivo. Distintas letras indican diferencias significativas en los valores medios entre tratamientos para un mismo tipo celular ($P < 0,05$). (*) El tiempo de exposición a 16 psu fue sólo de 10 días debido a la baja supervivencia.

within 4 h. No evidence of structural damage was observed in the midgut glands of these animals.

Only those shrimps abruptly transferred from 33 to 25 psu developed midgut gland lesions 96 h post-transfer to the lower salinity. Their midgut glands showed areas with necrotic foci, hemocytic infiltration and sloughing of epithelial cells into the lumen of the tubules. Tubule epithelium also displayed signs of hyperplasia and hypertrophy (Figs. 6 and 7). The midgut glands of the shrimps held at 33 psu and of those abruptly transferred from 33 to 29 psu presented a normal structure.

As in the first trial for the gradual salinity changes, there were no significant differences in mean heights of E-cells among treatments. There was a significant decrease in the mean height of F-cells of animals abruptly transferred from 33 to 25 psu for 24 h and those transferred for 96 h (from 28.5 ± 1.06 to 23.0 ± 0.90 µm, respectively) ($P < 0.05$) (Fig. 8a). Similarly, R-cells showed a significant decrease in mean height when comparing animals transferred from 33 to 25 psu for 24 h and those held for 96 h (from 29.1 ± 0.86 to 24.1 ± 0.77 µm, respectively) ($P < 0.05$) (Fig. 8b). B-cells were shorter in animals transferred from 33 to 25 psu for 96 h than in those held for 144 h (32.3 ± 1.19 to 38.6 ± 1.34 µm, respectively, $P < 0.05$) for which there were no significant differences with the control animals maintained at 33 psu (Fig. 8c). The

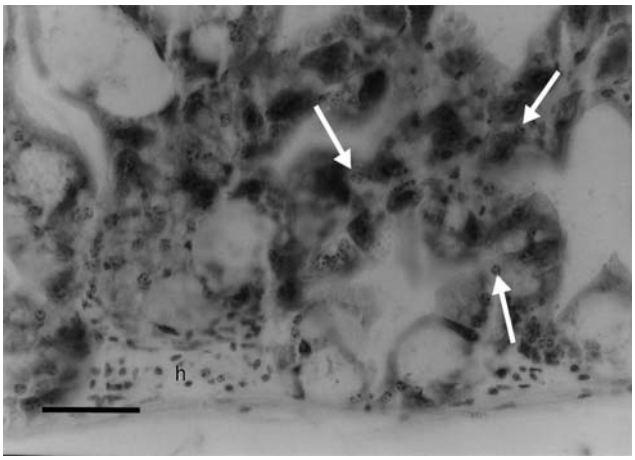

Figure 6. Hemocytic infiltration through the intertubule connective tissue of the midgut gland of *A. longinaris* maintained 96 h at 25 psu; H&E. Arrows show necrotic cells with karyorrhexic nuclei. h: hemocytes. Scale bar: 50 µm.

Figura 6. Infiltración hemocítica a través del tejido conectivo intertubular del hepatopáncreas de *A. longinaris* mantenidos 96 h a 25 psu; H&E. Las flechas muestran células necróticas con núcleos cariorréxicos. h: hemocitos. Escala: 50 µm.

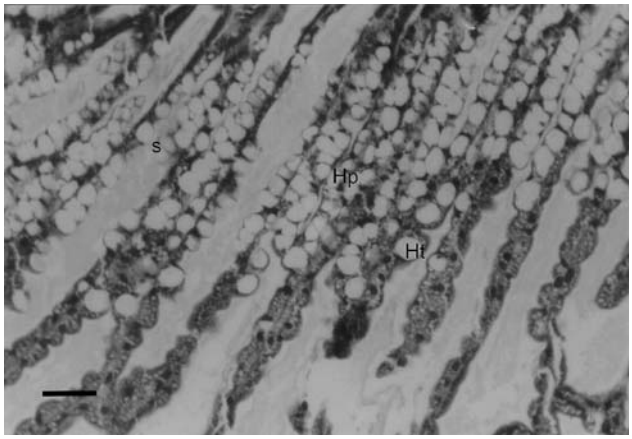

Figure 7. Longitudinal section of the midgut gland of *A. longinaris* maintained 96 h at 25 psu; hyperplasia, hypertrophy and sloughing of the tubule epithelium are evident; H&E. Hp: hyperplasia, Ht: hypertrophied cell, s: sloughing of tubule epithelium. Scale bar: 100 µm.

Figura 7. Sección longitudinal del hepatopáncreas de *A. longinaris* mantenidos 96 h a 25 psu; se observa hiperplasia, hipertrofia y descamación del epitelio de los túbulos; H&E. Hp: hiperplasia, Ht: célula hipertrofiada, s: descamación del epitelio del túbulo. Escala: 100 µm.

overall trend observed in Figure 8 is that F, R and B-cells reduced their height by 96 h and attained mean height values comparable to the control (33 psu, no salinity change) by 144 h.

DISCUSSION

Although it has been previously noted that *A. longinaris* does not tolerate low salinities (Harán *et al.*, 1992; D'Incao, 1999) there were no studies evidencing the histological changes in the midgut gland due to osmotic impairment. Gradual salinity changes from 33 to 25 and 29 psu does not affect survival and this was also reflected in the histological structure of the midgut gland which did not showed any alterations. In contrast, a gradual change from 33 to 16 psu was harmful, causing pleon necrosis and low survival (*i.e.*, a 10-day survival of 43%). Similarly, Harán *et al.* (1992) reported that below 25 psu survival and growth rates were greatly reduced, and that shrimps maintained at 16 psu developed muscular necrosis in their pleons and died. According to Lightner (1983), muscle necrosis appears after periods of severe stress (*e.g.*, overcrowding, low dissolved oxygen levels, abrupt temperature or salinity changes, rough handling, etc.), and it could be reversed if stress factors are reduced during its initial stages while large

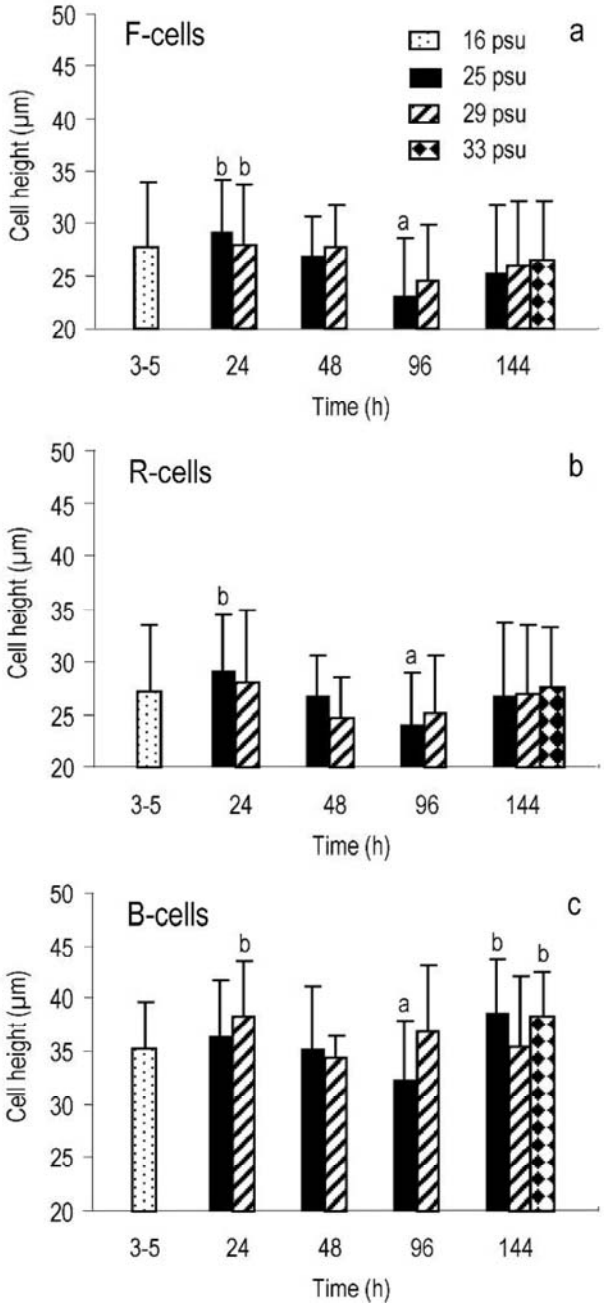

Figure 8. Mean heights (µm) of F (a), R (b) and B-cells (c) of *A. longinaris* suddenly transferred from 33 to 16, 25, 29 and 33 psu (no change) for different time intervals. Error bars represent the positive standard error. Different letters indicate statistical differences in mean values among treatments for a same cell type ($P < 0.05$).

Figura 8. Altura media (µm) de las células F (a), R (b) y B (c) de *A. longinaris* transferidos súbitamente de 33 a 16, 25, 29 y 33 psu (sin cambio) durante diferentes intervalos de tiempo. Las barras de error representan el error estándar positivo. Distintas letras indican diferencias significativas en valores medios entre tratamientos para un mismo tipo celular ($P < 0,05$).

areas of the pleon are still not compromised. Despite the low survival after 10 days at 16 psu, animals readapted to 33 psu recovered without noticeable sequels, swimming, eating and behaving normally.

The limited tolerance of *A. longinaris* to low salinities was confirmed by the pathologies observed, at the tissue level, in the midgut gland. A common histopathological sign in animals gradually exposed to 16 psu and held for 10 days was the sloughing of the epithelium of the tubules. Vogt (1990), explained that this pathological process occurs when cells are destroyed and other neighboring cells protrude like a wedge under the base of a damaged cell, extruding the necrotic cell into the tubular lumen while the neighboring cells simultaneously close the gap. When the number of destroyed cells becomes too high, as we observed in the tubules of shrimps held at 16 psu for 10 days, the gaps can no longer be closed and ulceration becomes evident. In addition, hemocytes invade the area, encapsulate the necrotic, cells often forming nodules, and release hydrolases that destroy the decaying tissue producing foci of necrosis like the ones observed.

From the abrupt salinity change experiment it can be deduced that even though death ensues in a few hours at 16 psu, there is not enough time for the midgut gland to develop observable histopathological signs. However, as also noted by Harán *et al.* (1992) the abdominal section of these animals became pale due to necrosis. The effect of an abrupt salinity change in the midgut gland of *A. longinaris* was detectable only after 96 h of being abruptly transferred from 33 to 25 psu. This salinity was the lowest at which animals survived long enough for the midgut gland to develop observable histological alterations.

Concerning the mean heights of F, R and B-cells in those animals gradually adapted from 33 to 16 psu, they were consistently smaller than those of animals gradually adapted from 33 to 29 psu ($P < 0.05$) and showed a mean value comparable to the control treatment when readapted to 33 psu for 30 days.

F, R and B-cells in animals abruptly transferred from 33 to 25 psu showed a tendency to decrease their mean height 96 h after transfer to this salinity and to recover a height comparable to the control treatment (33 psu, no salinity change) by 144 h after the transfer. These results suggest that it would take 96 h for a cell height change to become evident, and that by 144 h post-transfer, cells recover height values comparable to those of non-osmotically stressed individuals, thus indicating there could be an adaptation to the new osmotic conditions of the environment. Although the adaptation could be attributed to the osmotic change, the physiological processes that would cause it, are not known. E-cells were the only cell type that did not suffer significant changes in height at any of the salinities and exposure times tested. A study by Shires *et al.* (1994) on the structural changes of the gill cells of *Gammarus duebeni* caused by osmotic stress, concluded that following a salinity change, cell structure shows signs of osmotically imposed stress for a number of hours but returns towards a normal appearance within 10-16 h. Because of the internal location of the midgut gland, this organ may take longer to respond than the gills which are in direct contact with the stressor.

It is important to notice that although we didn't determine the intermolt stage of the shrimps at dissection, the cytological differences that occur at different stages in the molt cycle are due to the mobilization of reserves during the phases in which the animal does not eat (12 h before and 4 h after exuviation in this species) (Petriella, 1984). These differences can be detected by electron microscopy (Al-Mohanna & Nott, 1989).

The capacity of the midgut gland for healing and modifying its epithelium's height in response to osmotic changes supports the idea of an organ with high plasticity. It is already known to be used as an organ to monitor and detect adverse or stressful conditions in the environment. This is also supported by this study and contributes towards increasing the information on this species with aquaculture potential.

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