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Effects of Hypo- and Hypersaline Seawater on the Microanatomy and Ultrastructure of Epithelial Tissues of *Echinometra lucunter* (Echinodermata: Echinoidea) of Intertidal and Subtidal Populations

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Ivonete A. Santos-Gouvea and Carolina A. Freire (2007) Effects of hypo- and hypersaline seawater on the microanatomy and ultrastructure of epithelial tissues of *Echinometra lucunter* (Echinodermata, Echinoidea) of intertidal and subtidal populations. *Zoological Studies* **46**(2): 203-215. Echinoderms are widely distributed in intertidal zones and are thus subject to wide salinity variations and even air exposure. Physiological studies have shown them to be osmoconformers, but also to specifically, although moderately, regulate certain ions. Morphological studies associated with salinity challenges were not found in a literature search. Two populations of the sea urchin, *Echinometra lucunter* Linnaeus 1758, were studied: 1 intertidal and 1 subtidal population. Urchins from both populations were exposed for 5 d to hyposaline seawater (SW) of 25 ppt, or for 40 h to hypersaline SW of 45 ppt, and were compared to control urchins kept in full-strength SW of 35 ppt. Two external tissues, bathed by SW, the peristomial gills (PG) and ambulacral feet (AF), and 2 internal tissues, the coelomic wall of the ambulacral system (CWAS) and the intestinal rectum (IR), were investigated using transmission electron microscopy. With respect to the effect of salinity, there was more tissue damage in 45 ppt than in 25 ppt, coherent with the more-frequent SW dilution than concentration in marine shore habitats. Damage detected by electron microscopy included tissue disruption, destruction of microvillae, or evidence of fragmenting or peeling off of cells. Tissues of subtidal urchins were as sensitive to salinity stress as were those of intertidal urchins. PG, AF, and the IR presented evidence of an excretory function by the presence of large morula cells within these tissues. Only the IR displayed an ultrastructure compatible with metabolically active epithelia, in that it possessed numerous mitochondria. http://zoolstud.sinica.edu.tw/Journals/46.2/203.pdf

Key words: Ambulacral feet, Hypersaline seawater, Intestinal rectum, Peristomial gills, Water vascular system.

All members of the phylum Echinodermata are marine, considered stenohaline, and osmoconformers. Although they do not actively engage in osmoregulation, these invertebrates may regulate specific ions (Robertson 1949, Binyon 1962, Prusch 1977, Diehl 1986, Bishop et al. 1994). For example, transport of Cl^- has been related to nutrient uptake in the digestive tube of the sea urchin, *Lytechinus variegatus* (Bishop et al. 1994), and transport of K^+ to the lumen of ambulacral feet in starfishes has been related to the maintenance of internal pressure and volume through osmotic water flow (Prusch and Whoriskey 1976, Prusch 1977, Stickle and Diehl 1987). However, despite the fact that ionic regulation might not be a major feature of echinoderm physiology, these marine invertebrates are common dwellers of intertidal zones (McPherson 1969, Diehl 1986). As these habitats offer wide daily salinity fluctuations, at least some species must attain some degree of euryhalinity (e.g., Stickle and Diehl 1987).

Echinoderms also apparently do not possess any specific excretory organ (Hyman 1955, Boolootian 1966, Diehl 1986, Cavey and Märkel 1994); ammonia easily crosses the body wall, being the essential method of nitrogen excretion,

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as is typical of most aquatic animals. Ammonia diffusion occurs in the ambulacral feet and in the papullae of asteroids, as well as in the respiratory trees of holothuroids (Boolootian 1966). Non-diffusible metabolic wastes are actively secreted out of the body cavity, crossing rectal cecum cells and enterocytes into the intestinal lumen in echinoids, holothuroids, and asteroids (Warnau et al. 1998, Warnau and Jangoux 1999). These enterocytes display apical microvillae and a basal membrane associated with numerous elongated mitochondria (Warnau et al. 1998, Warnau and Jangoux 1999). There is also evidence of an excretory role of phagocytic coelomocytes associated with rectum enterocytes, the axial complex, ambulacral feet, and peristomial gills (Cobb and Sneddon 1977, Bachmann et al. 1980; Cavey and Märkel 1994). In addition to the evidence from enterocytes and the extrusion of phagocytic coelomocytes in several tissues, the axial gland has been demonstrated to display podocytes, a structure typically involved in ultrafiltration, which strengthens the idea that the excretory function is carried out not by a single excretory organ, but through a division of labor between several organs and tissues (Boolootian 1966, Cavey and Märkel 1994).

This study was conducted in order to: 1) evaluate the structural effects of hypo- and hypersaline stresses on echinoderm epithelial tissues, for the 1st time ever, looking at 2 ecologically distinct populations of urchins, 1 intertidal population, naturally subjected to daily tidal fluctuations in salinity, and 1 population inhabiting a stable subtidal environment; and 2) gather additional morphological evidence on the putative ion-transporting or excretory roles attributed to certain echinoderm tissues, by looking for abundant mitochondria and/or the presence of excretory cells. Two external tissues that are directly bathed by seawater, and 2 internal tissues were examined. The external tissues were the peristomial gills and adoral ambulacral feet, and the internal tissues were the coelomic wall of the ambulacral system and the intestinal rectum. The ultrastructures of echinoid tissues were previously described: the peristomial gills by Cobb and Sneddon (1977); the ambulacral feet and the channels and structures of the ambulacral system by Cavey and Märkel (1994); and the intestinal rectum by Warnau et al. (1998). The echinoderm model used here was the sea urchin, *Echinometra lucunter* Linnaeus 1758.

MATERIALS AND METHODS

Animals

An intertidal population of *E. lucunter* of Quilombo Beach, Penha (26°46'S, 48°38'W), Santa Catarina State, Brazil, was manually sampled from crevices and under rocks in tidal pools during low tide. The subtidal population was sampled through scuba diving off Galheta I., Pontal do Paraná (25°30'S, 48°15'W), Paraná State, Brazil, from a depth of 3-4 m. After being collected, adult male and female urchins of both populations were placed inside Styrofoam boxes with some water from the collection site and were transported to the laboratory. In the laboratory in Curitiba, they were maintained in a stock tank (160 L) containing sea water (SW) with a salinity of 35 ppt, a temperature of 20-24°C, constant aeration, and biological filtration. The average test diameter of the urchins of both populations used in this study was 77 mm.

Experiments

The control group of the intertidal population (4 urchins) was kept for ~16 hours in 35 ppt SW, being dissected the day after being collected in the field. All other urchins were kept for 3 d in the stock tank containing 35 ppt SW to allow them to acclimate to laboratory conditions. After that period, 2 animals were transferred and maintained in 30 L experimental aquaria, with constant aeration, pH control (7.5-8), a temperature of 22-24°C, biological filtration, and SW adjusted to a salinity of 35 ppt (controls for the subtidal population), 25 ppt (hyposaline SW), or 45 ppt (hypersaline SW). In order to increase the salinities, adequate amounts of marine salt were added. Evaporated marine salt was purchased from a local aquarium shop. Diluted SW was produced by appropriate dilution of full-strength SW with filtered (using activated charcoal and cellulose filters) tap water. Each experiment, for each of the 3 salinities and both populations, was performed in duplicate, always with 2 urchins per aquarium, yielding a total of 4 urchins for each salinity and each population. The only exception was for the subtidal population exposed to 25 ppt: only 3 urchins were used. Both intertidal and subtidal urchins in 25 ppt, and subtidal controls in 35 ppt were maintained for 5 d in the experimental aquaria. However, exposure of urchins from both populations to 45 ppt was for

only 40 h, due to morbidity and eventual mortality after 48-72 h in this concentrated, hypersaline SW. A 29% decrease (10 ppt, 35 to 25 ppt), and a 29% increase (10 ppt, 35 to 45 ppt) in salinity was the protocol employed, to allow a direct comparison between the responses of the urchins. The time of exposure to dilute SW (120 h, 5 d) was 3 times the time of exposure to hypersaline SW (40 h), evidencing the much-higher tolerance of the urchins to SW dilution compared to SW concentration.

Tissue dissection

After exposure to either the control or experimental conditions, urchins were anesthetized in magnesium chloride kept isosmotic to the control or experimental SW: 0.36 M $MgCl_2$ for 35 ppt, 0.26 M for 25 ppt, and 0.46 M for 45 ppt added to filtered tap water. After the spines had lost their tone, urchins were dissected for tissue removal. The external organs were removed first: 1 ambulacral foot from the oral hemisphere and a small branch of the peristomial gills. The urchin test was sectioned along its largest circumference using a fine hand saw; the distal most portion (~8 mm) of the intestine was removed from the aboral pole of the test; and finally some ampullae and canals of the ambulacral system of the oral hemisphere were taken.

Microanatomy and ultrastructure

A fragment (~2 mm^3) of each of the 4 tissues was immediately fixed by immersion in a primary fixative solution on ice for 2 h. The primary fixative solution contained 2.5% glutaraldehyde and 200 mM paraformaldehyde in 100 mM sodium cacodylate buffer, plus enough NaCl to render the fixative isosmotic to the tissues at each of the chosen salinities. This procedure was based on previous measurements of perivisceral coelomic fluid osmolalities, which confirmed the phenomenon of osmoconformation at the chosen exposure times and salinities. The coelomic fluid osmolality of urchins (from both populations) in 35 ppt was ~1047 mOsm/kg H_2O; after 5 d in 25 ppt, it was ~761 mOsm/kg H_2O; and after 3 d in 45 ppt, it was ~1433 mOsm/kg H_2O. For tissues from urchins maintained in 35 ppt, 0.3 M NaCl was added to the fixative. For tissues from urchins maintained in 25 and 45 ppt, 0.18 and 0.385 M NaCl were respectively added. Tissue fragments were then washed in cacodylate buffer and post-fixed in cacodylate buffer with the same NaCl concentrations as in the primary fixative, plus 1% osmium tetroxide for 1.5 h on ice. Fragments were dehydrated in an ethanol series, infiltrated, and embedded in Araldite 502 resin, using propylene oxide as the vehicle. All tissue fragments were oriented to allow transverse sections through the material. Semi-thin (500 nm) and thin (50 nm) sections were prepared with a Leica Ultracut UCT Ultramicrotome (Wetzlar, Germany). Semi-thin sections were stained with toluidine blue (1%), and photographed using a Zeiss Axiophot photomicroscope (Oberkochen, Germany). Thin sections were contrasted with 5% uranyl acetate and lead citrate (Reynolds 1963), and examined with a JEOL JEM 1200 EXII transmission electron microscope (Tokyo, Japan), at 80 kV of accelerating voltage.

Statistical analysis

Microanatomical dimensions were directly determined in print enlargements or in negatives of the micrographs, considering the respective total enlargement. Data from both populations were gathered, and control urchins in 35 ppt SW were compared to urchins in either 25 or 45 ppt SW, for an assessment of the effect of salinity on the specific shape and dimensions of tissues, using unpaired Student's *t*-test (Table 1); $p < 0.05$ was considered as statistically significant. Tissue damage (number of urchins showing tissue damage) under transmission electron microscopy was evaluated using the non-parametric Mann-Whitney test, $p < 0.05$, of urchins in 25 vs. 45 ppt SW (all tissues and both populations combined), intertidal vs. subtidal urchins (all tissues and both salinities combined), and external vs. internal tissues (both populations and both salinities combined).

RESULTS

Echinometra lucunter tolerated a SW salinity reduction from full-strength SW (35 ppt) down to 25 ppt, with no mortality detected after 5 d. When the salinity was raised by the same amount, a difference of 10 g/kg of salt (a 29% change) to 45 ppt, mortality occurred after 2-3 d of exposure. After 40 h in 45 ppt, all urchins were alive, but their spines and ambulacral feet had lost their tone, the peristomial membrane was retracted, and the teeth of the lantern were more exposed.

Microanatomy (by light microscopy)

Peristomial gills

Under a light microscope in cross section, the peristomial gills of *E. lucunter* are composed of an outer epithelium, bathed by SW, followed by connective tissue, and an inner/luminal epithelial layer bathed by peripharyngeal coelomic fluid, sometimes filled with coelomocytes (Figs. 1, 2). The circumference of the gills is very irregular, due to its arborescent shape, and depending on the site of the section, the lumen may appear empty or filled. When subjected to hyposaline SW (25 ppt), all specimens from both populations displayed a swollen lumen (Fig. 2). When subjected to hypersaline SW (45 ppt), all subtidal and half of the intertidal urchins displayed a dark material which entirely filled the lumen.

Adoral ambulacral feet

Under a light microscope in cross section, the ambulacral feet also possess an outer epithelium bathed by SW, a connective tissue layer, and an inner/luminal epithelium bathed by the fluid of the ambulacral system (Fig. 9). The circumference of the ambulacral feet of all control specimens, from both populations, was always very regular, being almost a circle. None of the controls displayed a lumen filled with coelomocytes or other cell material. All intertidal urchins in 25 ppt SW were similar to the control urchins. They only showed subtle evidence of edema. However, all subtidal urchins displayed evidence of edema in the connective tissues (Fig. 10). Still, the mean thickness of the ambulacral feet wall did not change between the controls in 35 ppt SW and urchins exposed to 25 ppt SW (p = 0.46; Table 1). Accordingly, the ratio of the thickness to the average diameter of the cross section did not differ between the controls and urchins in 25 ppt SW (p = 0.98; Table 1). None of the urchins from either population exposed to 25 ppt SW displayed lumen obstruction. However, all intertidal and three of 4 subtidal urchins subjected to hypersaline SW displayed abundant material inside the lumen and evidence of tissue disruption (Fig. 11). This result was confirmed by a quantitative assessment of the shape of the ambulacral foot cross section. In 45 ppt SW, both the thickness of the ambulacral foot wall (p = 0.023) and the ratio of the thickness to the average diameter increased (p = 0.009) when compared to values in 35 ppt SW (Table 1).

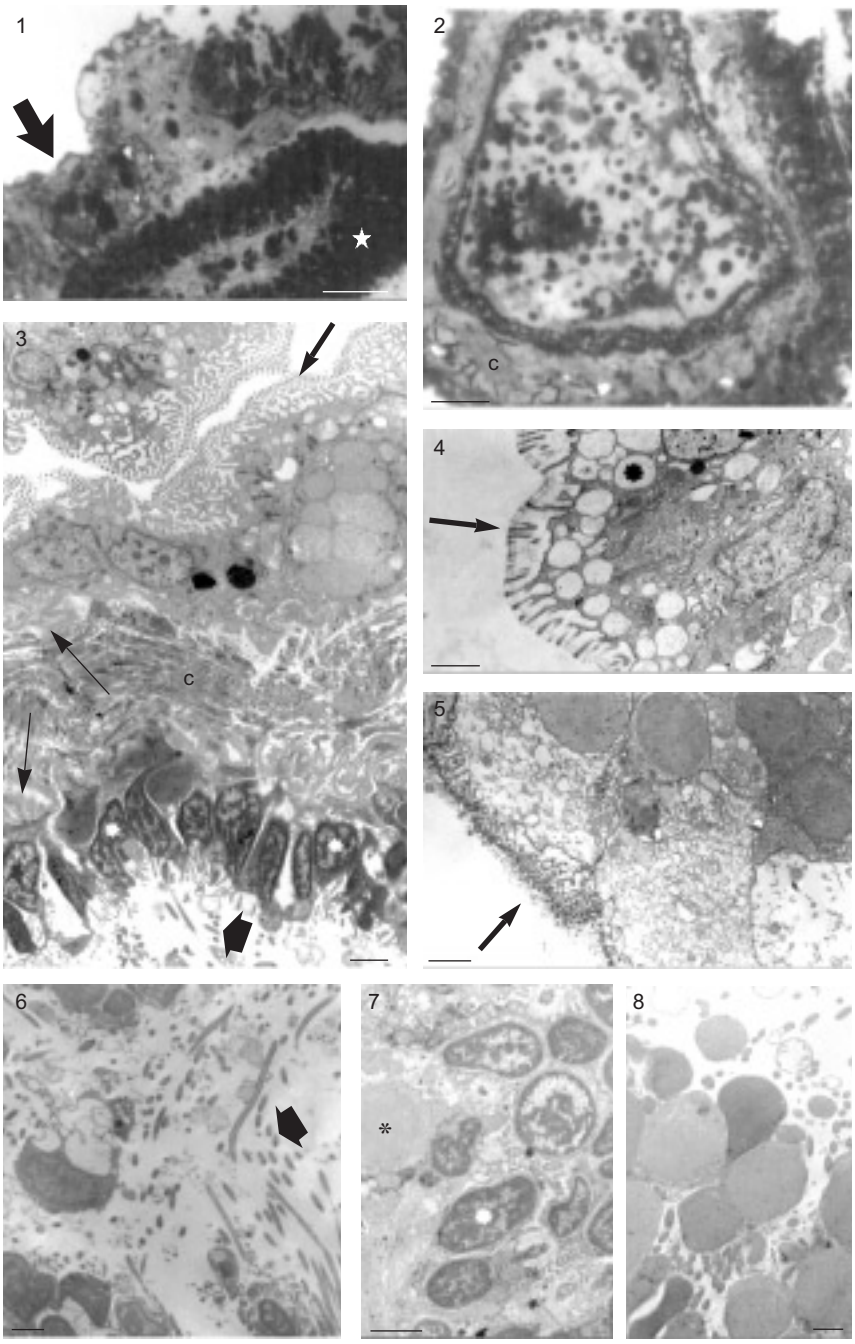
Coelomic wall of the ambulacral system

Light microscopy revealed the tissue layers of the coelomic wall that comprise the septate ampullae and channels of the ambulacral system. This

Table 1. Summary of microanatomical dimensions observed under light microscopy in intertidal or subtidal populations *Echinometra lucunter* in the control salinity of 35 ppt, or submitted to hypo- (25 ppt) or hypersaline (45 ppt) seawater. Data from the intertidal and subtidal populations were combined to increase the number of samples (n) given in parentheses

	Dimensions (mean ± SEM)					
	External tissue		Internal tissues			
Salinity	Ambulacral feet		Coelomic wall		Intestinal rectum	
	Wall (μm)	Wall/ diameter	Wall (μm)	Wall/diameter	Epithelium (μm)	Strip of vesicles (μm)
35 ppt	113 ± 12.8 (8)	0.23 ± 0.02 (8)	18.3 ± 2.8(6)	0.17 ± 0.02(6)	218 ± 28.1(7)	35.3 ± 6.1(8)
25 ppt	136 ± 28.5 (7)	0.23 ± 0.01 (6)	21.5 ± 4.0 (6)	0.20 ± 0.03 (6)	202 ± 13.5 (5)	54.2 ± 9.2 (5)
45 ppt	181 ± 25.0* (6)	0.33 ± 0.03* (6)	18.7 ± 3.3 (6)	0.20 ± 0.04 (6)	174 ± 15.6 (7)	28.5 ± 3.1 (6)

*p < 0.05, compared to the controls in 35 ppt seawater.



Figs. 1-8. Microanatomy and ultrastructure of the peristomial gills of *Echinometra lucunter* in cross section. **1.** Light micrograph (LM) of the peristomial gills from a control intertidal urchin in 35 ppt seawater (SW), showing the irregular outer shape of a transverse section through the arborescent peristomial gill, its outer epithelium (⬅), and thick inner epithelium (★, white). **2.** LM of the peristomial gills from a subtidal urchin in 25 ppt SW, showing abundant material inside the lumen, and a thick connective tissue layer (c). The shape of the gills is rounded, and the lumen is enlarged probably due to water entry. **3.** Electron micrograph (EM) of the peristomial gills from an intertidal urchin in 25 ppt SW, showing a panoramic view of the tissue, extending from the outer epithelium with microvillae (➡) to the inner (luminal) epithelium with cilliated cells (➡). Connective tissue (c) can be observed between the basement membrane of both epithelia (⟶). **4.** EM of the peristomial gills from a subtidal urchin in 25 ppt SW showing microvillae preservation in the outer epithelium (➡) and abundant mucous vesicles (✹). **5.** EM of the peristomial gills from a subtidal urchin in 45 ppt SW showing the outer epithelium with damaged microvillae (➡). **6.** EM of the peristomial gills from a control intertidal urchin in 35 ppt SW showing the inner ciliated epithelium (➡). **7.** EM of the peristomial gills from a subtidal urchin in 25 ppt SW showing muscle cells (✱) between the luminal epithelial ciliated cells (✹, white). **8.** EM of the peristomial gills from an intertidal urchin in 45 ppt SW, showing the inner luminal epithelium damaged by the exposure to hypersaline SW, with cells peeling off. Scale bars: LM, 50 μm; EM, 2 μm.

tissue has an outer epithelium bathed by perivisceral coelomic fluid, a connective tissue layer, and an inner/luminal epithelium bathed by the fluid of the ambulacral system. Control urchins from both populations (e.g., intertidal Fig. 18) displayed ampullae with their interseptum space either empty or filled with material. Urchins in 25 ppt SW did not essentially differ from control urchins with respect to the microanatomy of their ampullae under light microscopy. The interseptum spaces were, as in the controls, either empty or filled with material. Occasionally urchins displayed evidence of edema with fluid between the 2 epithelia but with no apparent sign of tissue disruption. The tissues of all urchins submitted to hypersaline SW were well preserved, with the exception of a single intertidal urchin which displayed tissue disruption. Contrary to what was detected in the ambulacral feet, neither the thickness of the wall nor the shape of the channels (the ratio of the wall thickness to the average diameter) was affected by exposure to either a reduction or increase in salinity ($p > 0.05$ for all comparisons with the controls at 35 ppt) (Table 1).

Intestinal rectum

The intestinal rectum of *E. lucunter* is composed of an outer epithelium (bathed by the perivisceral coelomic fluid) next to a connective tissue layer, and a thick inner, luminal epithelium (Fig. 21). Abundant large cells rich in cytoplasmic vacuoles were observed within the layers of the rectal tissue (Fig. 21). These cells were identified as morula cells after the electron microscopic analysis. The edge of the luminal epithelium displayed a thick well-defined strip with clear vesicles of a seemingly mucosal nature (Fig. 21). Intertidal and subtidal urchins submitted to 25 and 45 ppt SW also presented this luminal strip of vesicles. The perivisceral epithelium was well preserved in all experimental animals, both in 25 and 45 ppt SW. As a consequence, the average thickness of the epithelium of the strip of clear vesicles did not change upon exposure to either hypo- or hypersaline SW ($p > 0.05$ for all comparisons with controls in 35 ppt) (Table 1).

Ultrastructure (by transmission electron microscopy)

Peristomial gills

The ultrastructure of the outer epithelium of the peristomial gills (bathed by SW) of both intertidal and subtidal urchins displayed epithelial cells with microvillae, and some subapical vesicles were filled with material of low electron density (Figs. 3, 4). Sparse mitochondria were also observed, in the subapical region. Right below the epithelium, a basal membrane was observed. A layer of connective tissue mixed with muscle cells was observed between the basal membrane of the external epithelium and the basal membrane of the internal epithelium. Some large cells with variable locations and section shapes and sizes were observed in the peristomial gills of *E. lucunter*. Their cytoplasm was entirely filled with vacuoles containing amorphous material of variable electron densities. They are hereafter treated as morula cells, the same cells mentioned above, detected under light microscopy in the intestinal rectum of *E. lucunter* (Boolootian 1966, Bachmann and Goldschmid 1978, Motokawa 1982, Cavey and Märkel 1994). There was no notable difference between the controls of the 2 populations. The luminal epithelium bathed by peripharyngeal coelomic fluid revealed layers of ciliated cells, with little cytoplasm and a large nucleus (Figs. 3, 6, 7). Muscle cells occurred between these epithelial cells, yielding a myoepithelium (Fig. 7). No difference with respect to the controls was elicited by exposure of urchins from both populations to 25 ppt SW (Figs. 3, 4, 7). However, hypersaline SW led to damage to the microvillae (of the outer epithelium) (Fig. 5, Table 2). In 45 ppt SW, urchins frequently displayed a damaged inner epithelium, with cells apparently peeling off (Fig. 8, Table 2).

Adoral ambulacral feet

The outer epithelium of the ambulacral feet of the control (35 ppt) intertidal and subtidal urchins revealed epithelial cells with microvillae and numerous subapical vesicles (Fig. 12), similar to peristomial gills. Large morula cells were frequently observed (Figs. 12, 15). These large cells were observed in all control urchins from both populations and were distinctly more frequent in the ambulacral feet than in the peristomial gills. Mucous cells were also observed (Figs. 12, 15). The luminal epithelium, bathed by the ambulacral fluid, revealed a thick myoepithelium apposed to the basal membrane (Fig. 13). Neurons were localized between these podial myocytes, displaying vesicles with neurotransmitters (Fig. 17). Small ciliated support cells were observed lining the lumen, with a few cilia and a small volume of

cytoplasm (Figs. 13, 14). Organized circular and longitudinal collagen bundles were present below the basal membrane (Fig. 13). Exposure to SW dilution produced no differences in the ultrastructure with respect to the controls for either intertidal or subtidal urchins, for both the outer and inner epithelia (Figs. 14, 15, Table 2). Morula cells were also observed in urchins exposed to 25 (Fig. 15) and to 45 ppt SW. Some of the subtidal urchins in 45 ppt SW (Table 2) had damaged epithelial cells and microvillae (Fig. 16). Tissue edema was observed, with muscle cells spaced widely apart from each other (Fig. 14) in 25 ppt SW, but this was not considered serious tissue damage (Table 2). All urchins exposed to 45 ppt SW, both intertidal (Fig. 17) and subtidal ones, exhibited cell precipitation into the lumen of their ambulacral feet with cells peeling off (Table 2).

Coelomic wall of the ambulacral system

When examined with transmission electron microscopy, the ampullae of control urchins kept in 35 ppt SW showed that the epithelium lining the coelomic cavity (outer epithelium) is composed of a layer of cells displaying a few cilia. These slender cells displayed an irregularly shaped nucleus and little cytoplasm, and thus are supporting cells. Loose connective tissue was found between the basal membranes of the outer and inner epithelia (Fig. 19). The luminal myoepithelium was bathed by the fluid of the ambulacral system (Fig. 19). Epithelial cells of this luminal inner myoepithelium are ciliated, and the cilia were frequently surrounded by microvillae. In 25 ppt SW, half of the intertidal urchins (2/4) and most subtidal (2/3) ones exhibited a wider connective tissue strip, with evidence of edema, but this was not considered damage (Table 2). Again, more tissue damage was detected in 45 ppt SW in subtidal urchins (Fig. 20, Table 2) with cells peeling off. Most intertidal urchins were similar to the controls.

Intestinal rectum

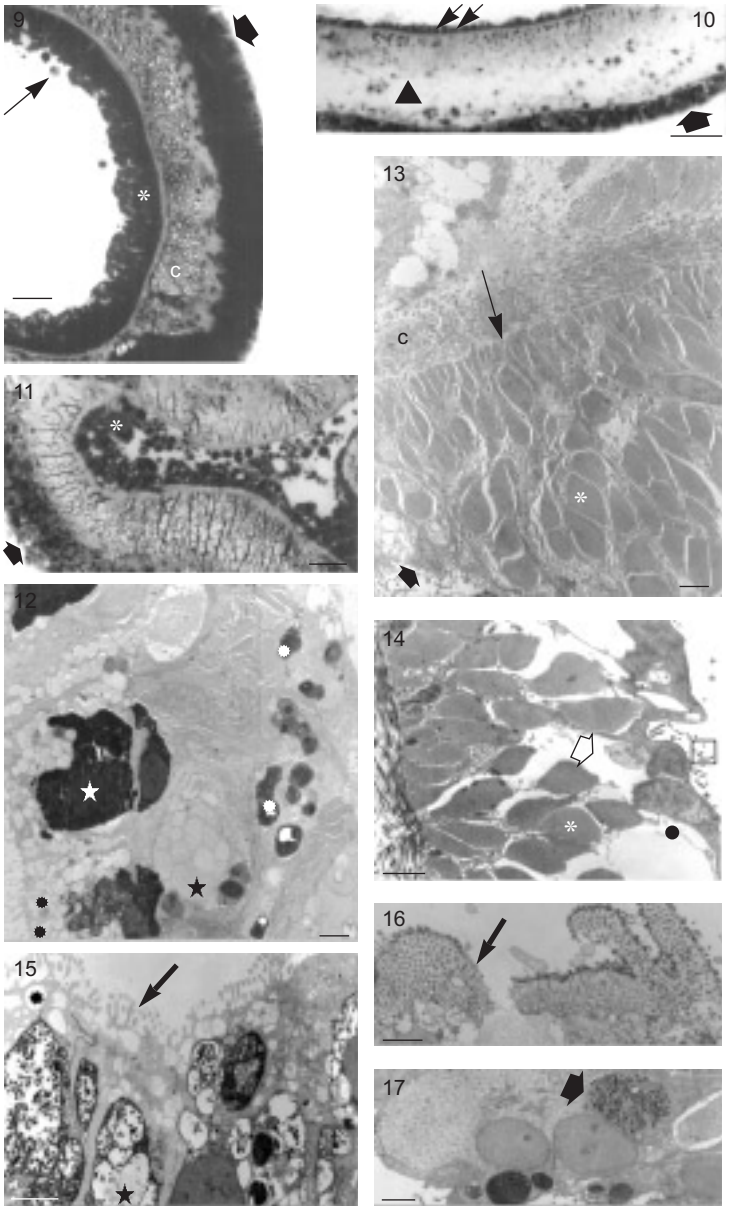
The electron micrographs show microvillae in the luminal epithelium, as well as abundant mucous vesicles of low electron density (Figs. 22, 23) pictured in the light micrograph (Fig. 21). Numerous mitochondria were also observed (Figs. 22, 23). Between the epithelial cells, large vacuolated morula cells (Figs. 23, 24), also seen under light microscopy in this tissue (Fig. 21), were very abundant, both in control and experimental urchins. Exposure to 25 ppt SW (Fig. 23) led to less frequent damage to microvillae than did exposure to 45 ppt SW (Fig. 25, Table 2).

Analysis of damage data under transmission electron microscopy (Table 2)

Exposure to hyposaline SW (25 ppt) led to less-frequent ultrastructural damage than exposure to hypersaline SW (45 ppt) ($p < 0.001$), but

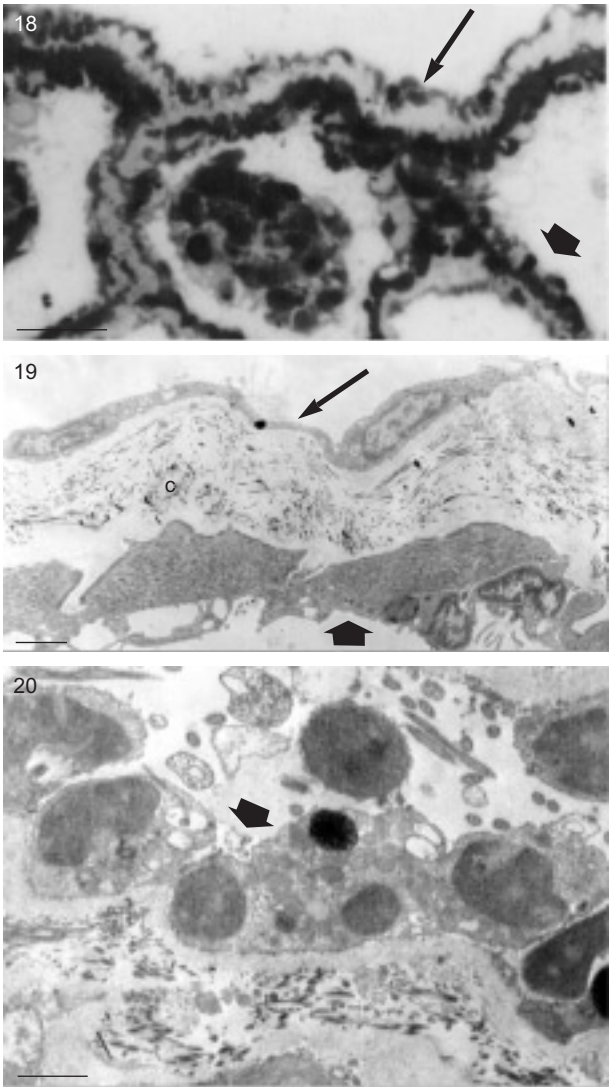
Table 2. Summary of tissue damage detected under transmission electron microscopy induced by hypo- (25 ppt) or hypersaline (45 ppt) seawater (SW) exposure of intertidal and subtidal *Echinometra lucunter* populations. In 25 ppt SW, damage consisted of damaged microvillae in intestinal rectum. In 45 ppt SW, damage consisted of cells peeling off of the inner epithelium (ie) and damaged microvillae in the outer epithelium (oe) in the peristomial gills and ambulacral feet; cells peeling off of the coelomic wall of the ambulacral system; and damaged microvillae or cells peeling off of the intestinal rectum

		Number of urchins showing tissue damage					
		External tissues				Internal tissues	
Salinity	Population	Peristomial gills		Ambulacral feet		Coelomic wall	Intestinal rectum
		ie	oe	ie	oe		
25 ppt	Intertidal	0	0	0	0	0	1
	Subtidal	0	0	0	0	0	0
45 ppt	Intertidal	1	2	4	0	1	2
	Subtidal	4	1	4	2	4	2



Figs. 9-17. Microanatomy and ultrastructure of the ambulacral feet of *Echinometra lucunter* in cross section. **9.** Light micrograph (LM) of an ambulacral foot of a control intertidal urchin in 35 ppt seawater (SW), showing good tissue preservation and the expected regular shape of a cross section of the foot, connective tissue (c), inner (luminal) epithelium (✱, white), loose coelomocytes in the lumen (➞), and outer epithelium (➡). **10.** LM of the wall of an ambulacral foot of a subtidal urchin in 25 ppt SW, showing less-compact and -swollen connective tissue (▲), inner epithelium (➞), and outer epithelium (➡). **11.** LM of an ambulacral foot of a subtidal urchin in 45 ppt SW showing the irregular shape and altered outer epithelium (➡), and fragmentation of the loose inner epithelium (✱, white). **12.** Electron micrograph (EM) of an ambulacral foot of a control intertidal urchin in 35 ppt SW showing morula cells with dark, electron-opaque concretions (★ and ✹, white), cells with mucous content (★), and abundant vesicles (✹) in the outer epithelium with microvillae. **13.** EM of an ambulacral foot of a control subtidal urchin in 35 ppt SW showing the thick myoepithelial layer (✱, white) apposed to the basement membrane (➞) and connective tissue layer (c). **14.** EM of an ambulacral foot of a subtidal urchin in 25 ppt SW showing luminal ciliated (□) epithelial cells (●), muscle cells (✱, white), and spacing between the cells due to water entry (edema, ⇨). **15.** EM of an ambulacral foot of a subtidal urchin in 25 ppt SW showing morula cells (★) between the outer epithelial cells with microvillae (➞), and abundant vesicles (✹). **16.** EM of an ambulacral foot of a subtidal urchin in 45 ppt SW showing damaged microvillae (➞). **17.** EM of an ambulacral foot of an intertidal urchin in 45 ppt SW showing damaged luminal epithelium with cells peeling off or fragmenting, and a neuron with vesicles (➡). Scale bars: LM, 50 μm; EM, 2 μm.

subtidal urchins were equally sensitive to the saline stresses imposed as were intertidal urchins (p = 0.664), and the external tissues (peristomial gills and ambulacral feet) were as often damaged as internal tissues (coleomic wall of the ambulacral system and intestinal rectum) (p = 0.798).



Figs. 18-20. Microanatomy and ultrastructure of the coelomic wall epithelia of the ambulacral system of *Echinometra lucunter* in cross section. **18.** Light micrograph of the coelomic wall of the ambulacral system from a control intertidal urchin in 35 ppt seawater (SW), showing the outer perivisceral epithelium (➞) and the inner (luminal) epithelium that faces the ambulacral fluid (➡); note that the lumen is filled with coelomocytes. **19.** Electron micrograph (EM) of the coelomic wall of the ambulacral system of a control subtidal urchin in 35 ppt SW, showing the outer epithelium (➞) and inner (➡) myoepithelium and connective tissue layer (c). **20.** EM of the coelomic wall of the ambulacral system of a subtidal urchin in 45 ppt SW, showing a fragmented luminal epithelium (➡). Scale bars: LM, 50 μm; EM: 2 μm.

DISCUSSION

Salinity effects: hypo- vs. hypersaline stress

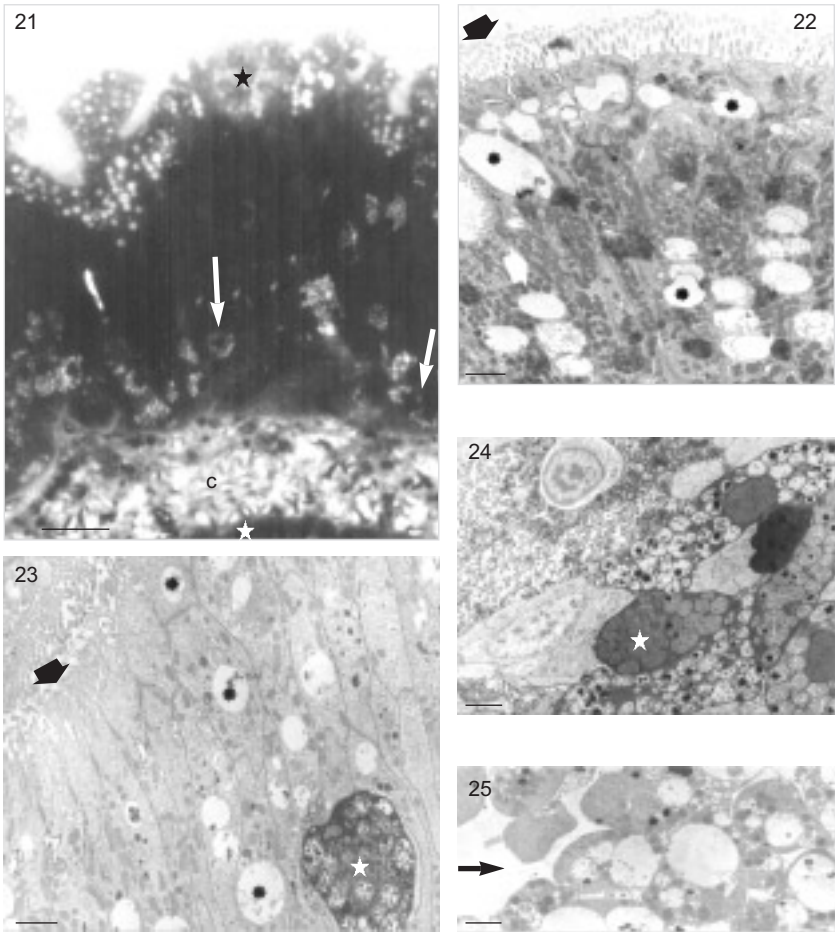
Both populations survived well in hyposaline SW of 25 ppt, but not in hypersaline SW of 45 ppt. While 10 ppt of a salinity decrease was perfectly tolerable, a salinity increase by the same amount (a 29% change) presented an unbearable stress, with mortality after 48-72 h. Urchins of the same species submitted to SW of salinities of 20, 13, and 7 ppt displayed loss of movement after 2-24 h (Gomes 1973). Mortality in hypersaline SW probably resulted from significant osmotic water loss, with an inability of the cells to regulate volume (RVI), and consequent ultrastructural damage. Microanatomical alterations in hypersaline SW were also observed under light microscopy, mostly in the peristomial gills and ambulacral feet, with abundant material filling the lumen and an alteration in the thickness of the wall of the ambulacral feet. A higher capacity to regulate the cell volume after osmotic water entry than after osmotic water loss is indeed expected (Diehl 1986), as in their intertidal habitat, SW dilution is much more likely than its concentration (Diehl 1986, Stickle and Diehl 1987). In any case in the laboratory, *E. lucunter* tolerated a fairly steeper salinity challenge than it would ever likely encounter in nature, even in an intertidal habitat.

Urchins from the 2 populations did not significantly differ in their sensitivities to the saline stresses imposed. Although intertidal urchins face daily salinity or desiccation challenges during tidal cycles, and subtidal urchins live in a rather-stable environment, the saline stresses imposed here did not produce any significant interpopulational differences in the ultrastructural damage quantified. Perhaps a different protocol, with verification of survival under gradual SW dilution or concentration could reveal interpopulational differences. Indeed, the starfish (*Asterias rubens*) and sea urchin (*Strongylocentrotus droebachiensis*) obtained from low-salinity areas were more tolerant of salinity reduction when compared to specimens collected in deeper, subtidal areas (Topping and Fuller 1942).

Salinity effects: external vs. internal tissues

The 4 tissues of *E. lucunter* examined herein displayed no marked differences in their sensitivities to the salinity stresses imposed, independent of the population. Peristomial gills and ambulacral

feet are external structures, forming interfaces between the body of the animal and SW. On the other hand, the coelomic wall of the ampullae and canals of the ambulacral system are bathed externally by the perivisceral coelomic fluid, and internally by the fluid of the ambulacral system. The intestinal rectum is also bathed by the perivisceral coelomic fluid externally and by the intestinal fluid internally. These last 2 tissues (the ampullae and rectum) are internal organs not directly exposed to SW. However, there was no significant difference in the sensitivity to the harmful effects of salinity (mainly hypersaline SW) on the external tissues and organs, when compared to the internal structures. The harmful effects considered here were damaged microvillae and ciliated myoepithelium cells which seemingly were fragmenting or peeling off (Table 2). This would indeed be an expected outcome, as these osmoconforming sea urchins (Vidolin 2003) are expected to be highly permeable to water and ions (Diehl 1986), with the outcome of gradients dissipating in less than 24 h (Diehl 1986, Stickle and Diehl 1987); actually, after 6 h (Vidolin 2003). In this way, internal structures would be expected to suffer as much of an effect from the saline stresses imposed as would the external structures, since all of them were analyzed after 5 d (at 25 ppt) or 40 h (at 45 ppt). Nevertheless, under light microscopy, the internal structures of the urchins in 45 ppt SW displayed



Figs. 21-25. Microanatomy and ultrastructure of the intestinal rectum of *Echinometra lucunter* in cross section. **21.** Light micrograph of the rectum from a control subtidal urchin in 35 ppt seawater (SW), showing morula cells (➞, white), a strip of apical mucous vesicles (★) in the luminal epithelium, and the connective tissue layer (c). **22.** Electron micrograph (EM) of the rectum from a control intertidal urchin in 35 ppt SW, showing microvillae in the luminal epithelium (➡), numerous mitochondria (➡, white), and apical mucous vesicles (✹). **23.** EM of the rectum from a subtidal urchin in 25 ppt SW, showing morula cells (★, white) and mucous vesicles (✹) in the apical region of the luminal epithelium with microvillae (➡). **24.** EM of the rectum from a subtidal urchin in 25 ppt SW showing morula cells (★, white). **25.** EM of the rectum of a subtidal urchin in 45 ppt SW showing damaged luminal epithelium, without evident microvillae (➞). Scale bars: LM, 50 μm; EM, 2 μm.

less change than did the external structures when compared to the controls. This result may be a consequence of a certain damping effect of the perivisceral coelomic fluid, which can delay the deleterious effects of hypersalinity on the internal tissues (Stickle and Ahokas 1974). Indeed, *E. lucunter* (Vidolin 2003) and *Lytechinus variegatus* (Bishop et al. 1994) display some small but significant ionic gradients between the coelomic fluid and external SW. Additionally, osmotic and/or ionic differences between echinoderm internal fluids have also been reported (Diehl 1986). Ion-transporting epithelia supposedly display abundant mitochondria (Warnau et al. 1998, Warnau and Jangoux 1999). In the present study, only the intestinal rectum (Warnau et al. 1998) showed abundant mitochondria. This result confirms the apparent central role of the echinoid intestinal rectum in transepithelial transport, although it does not allow one to conclude anything about the nature of the molecules/substances transported.

Peristomial gills, morula cells, and excretion

The capacity to specifically regulate ions and sustain ionic gradients is certainly not the only unsettled issue in echinoderm physiology. The function of the peristomial gills of sea urchins is also controversial. The circulation of the peripharyngeal fluid in the lumen of peristomial gills is dependent upon the movement of Aristotle's lantern, generating fluid flow inwardly and outwardly through the peristomial gills (Hanson and Gust 1986, Cavey and Markel 1994). Despite being called "gills", their function in gas exchange was already questioned decades ago (Cobb and Sneddon 1977, Cavey and Märkel 1994). The peristomial gills represent a small portion of the surface of the animal, when compared, for instance, with the ambulacral feet (Cobb and Sneddon 1977, Cavey and Märkel 1994). Additionally, removal of the peristomial gills does not significantly modify oxygen uptake, and the peristomial gills face the substrate, a region of limited water circulation (reviewed in Cavey and Markel 1994). Another factor that apparently limits gas exchange through the peristomial gills is that the tissue is rather thick, as observed in figures 1-8, and this is not ideal for rapid gas equilibration. Because of these factors, the name "peristomial gills" might appropriately be changed to "peristomial diverticula", and their function may well be more excretory than respiratory.

The large vacuolated cells called morula cells were found in several tissues of *E. lucunter*. Not only in the peristomial gills, but also and even more abundantly in the ambulacral feet and intestinal rectum. They are morphologically compatible with the description and depiction of morula cells from the literature (Boolootian, 1966, Bachmann and Goldschmid 1978, Motokawa 1982, Cavey and Märkel 1994). The visualization of these cells within the tissues of *E. lucunter* suggests cellular traffic between the coelom and the external medium, compatible with an excretory function. These morula cells might thus be the same excretory cells called "necrotic", from the peristomial gills of *Echinus esculentus* (Cobb and Sneddon 1977). A precise identification of the contents of the vacuoles of the morula cells will have to await future studies, although their appearance as late phagocytes lends support to the contention of their excretory role.

Microvillae and putative mucous secretion

The outermost layers of the peristomial gills and ambulacral feet of *E. lucunter*, bathed by external SW and covered with microvillae, are like those described for other urchins (Fenner 1973, Cobb and Sneddon 1977, Cavey and Märkel 1994). Microvillae confer increased surface area, suggesting that these epithelial cells may transport certain substances to or from SW (Coleman 1969). The epithelial cells displayed vesicles of low electron density, and occasionally appeared to be fused with the external membrane/microvillae. Those vesicles probably contain mucous granules that build a "cuticle" when externalized, for protection (Coleman 1969, Santos and Sasso 1970, Florey and Cahill, 1977, Hajduk 1992). Actually, the presence of microvillae in the external epithelia of peristomial gills and ambulacral feet may rather be a byproduct of the intense exocytosis of mucous granules than increased membrane area for absorption of any molecule in the 1st place. This protective role played by mucous secretion in the outer epithelia has also been detected in the intestinal rectum of *E. lucunter*. Mucous secretion protects the luminal cells, making it easier for the digestive material to progress longitudinally and also making it more difficult for unwanted substances to cross the epithelium (Holland and Ghiselin 1970). Judging from the abundance of mitochondria and morula cells, the posteriormost part of the intestine of *E. lucunter* is apparently intensely involved in excretory transport (Warnau et al. 1998, Warnau and Jangoux 1999).

In conclusion, both populations were more sensitive to hypersaline seawater than to hyposaline seawater, with no significant difference between the 2 populations detected. A higher capacity to regulate the cellular volume when facing osmotic water entry was thus evident, as reported in the literature (Diehl 1986). Consistent with the high general body wall permeability expected for these marine invertebrates displaying osmoconforming behavior, the internal structures and organs in both populations displayed as much tissue damage from saline stresses than did the external ones under the protocol employed here. Additionally, of the 4 tissues examined, only the intestinal rectum displayed mitochondria in a density compatible with active transepithelial transport. The rectum may thus also have an excretory function through the putative extrusion by morula cells, sharing this last function with other tissues, such as the peristomial gills and ambulacral feet.

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