# The morphometric changes in the gills of the estuarine crab *Chasmagnathus granulatus* under hyper- and hyporegulation conditions are not caused by proliferation of specialised cells

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ABSTRACT

*Chasmagnathus granulatus* is a hyper-hyporegulating crab that inhabits changing habitats of salinity in Brazil, Uruguay and Argentina. Since the gills are the main sites for active ion transport in crabs, the adaptive changes in the gill epithelium occurring under different conditions of salinity were studied by means of morphological and morphometric analysis, and immunohistochemical identification of cell proliferation (BrdU technique). In anterior (1–3) gills the epithelium thickness from crabs acclimatised to 12, 34 and 44 g/l ranged from 1.27 to 2.46 μm, with no significant change during acclimatisation, thus denoting a respiratory function. Medial (4–5) gill epithelium was slightly thicker in extreme salinities, but these differences were not statistically significant. In contrast, epithelial thickness of the posterior (6–8) gills increased significantly up to 8.10 μm (dorsal zone of gill 8) both in hyper- and hyposaline media compared with seawater. The dark areas measured in gill 8 treated with $AgNO_3$ revealed putative ion transporting tissue, especially at 12 and 44 g/l, corresponding to the zones of higher epithelial thickness. Hence these areas seem to participate both in hyper- and hyporegulation. Proliferating cells labelled with BrdU almost never occurred in the gills/salinity combinations studied during the initial 48 h of transfer from seawater to hyperconcentrated or diluted media, thus suggesting an increase in cell size rather than cell proliferation.

*Key words*: Ion-regulation; salinity; Crustacea.

## INTRODUCTION

Estuarine crustaceans are able to tolerate pronounced variations in external salinity. Most of these species possess ion hyperregulatory mechanisms which allow them to exploit dilute seawater environments efficiently (Mantel & Farmer, 1983). Species showing strong hyperregulation capacities are able to colonise low salinity or even freshwater environments. On the other hand, the hyper-hyporegulation capacity is characteristic of semiterrestrial and terrestrial crabs (excluding 'freshwater-land crabs'), species able to maintain fairly constant levels of ionic variables both at low and high salinities (Gross, 1957; Schubart & Diesel, 1998, 1999). Examples of this capacity are ocypodid and grapsid crabs such as *Goniopsis cruentata*, *Uca* sp., *Pachygrapsus marmoratus* and *Chasmagnathus granulatus* (Rabalais & Cameron, 1985; Martelo & Zanders, 1986; Pierrot et al. 1995; Mougabure Cueto, 1998).

Crab gills have been described as participating not only in respiratory gas exchange, but also in acid-base balance, ion regulation and ammonia excretion. The anterior gills are the main sites for gas exchange whereas the posterior ones participate mainly in salt transporting processes (Siebers et al. 1982; Gilles & Péqueux, 1986; Towle, 1990; Taylor & Taylor, 1992; Weihrauch et al. 1998). The epithelium lining posterior gills characteristically shows thick cells with extensive interdigitations of the plasma membranes closely related to mitochondria (Copeland, 1968). Several ATPases and ionic exchangers have been reported to be located in the membranes of these cells, being involved in active salt uptake together with

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these mitochondria (see Lucu, 1993; Péqueux, 1995, for reviews).

There are several studies on ultrastructural changes in the gill epithelium of euryhaline crabs in relation to external salinity (Finol & Croghan, 1983; Martelo & Zanders, 1986; Compère et al. 1989; Luquet et al. 1997; McNamara & Lima, 1997). However no morphometric analysis of the branchial epithelium of crabs related to these salinity-induced changes has so far been carried out. At present, there are 2 hypotheses to explain the reported salinity-induced changes in the gill epithelium. (1) Cell turnover: cells specialised in ion uptake are replaced by new (proliferating) cells with ion excreting capacity when the external medium shifts from hypo- to hyperconcentrated, as suggested by Wendelaar Bonga & Van Der Meij (1989) and Van Der Heijden et al. (1997) for the euryhaline fish *Oreochromis mossambicus*. (2) Cell plasticity: the gill epithelial cells specialised in ion uptake at low salinity undergo ultrastructural changes that confer upon them the capability to excrete ions when the external medium becomes hyperconcentrated.

The brachyuran crab *Chasmagnathus granulatus* inhabits estuary zones of Brazil, Uruguay and Argentina, where salinity medium constantly changes both in a tidal and on a seasonal basis. Animals of this species can also be found moving actively to supratidal zones and are thus exposed to highly concentrated water. The euryhaline condition of this crab ensures its distribution from marine coasts to the interior part of estuaries, where salinity can be lower than 1 g/l at low tide. *C. granulatus* has been reported as keeping its internal concentrations of sodium, chloride and potassium similar to the external medium when water salinity is about full strength seawater. At lower salinities, e.g. 12 g/l, this species strongly hyperregulates while at higher salinities e.g. 44 g/l, it hyporegulates at levels between 73–82% of the medium concentration (Luquet et al. 1992; Mougabure Cueto, 1998). Previous work has demonstrated that the ultrastructure of the epithelial cells lining the posterior gills of *C. granulatus* changes according to the salinity, the cell morphology being clearly modified 48 h after transference (Rosa et al. 1999).

The aim of this study was to obtain morphometric evidence on the participation of the gills in the hyporegulatory processes as well as in hyperregulation. It was also designed to examine, by a cell proliferation assay, the hypotheses of cell turnover and cell plasticity for explaining the ultrastructural differences between gills from low and high salinity adapted crabs.

## MATERIALS AND METHODS

### *Animals*

Individuals of *C. granulatus* were collected from the southern end of Samborombón Bay (36° 18′ S, 56° 48′ W), Buenos Aires province, Argentina. In the laboratory, 45 adult intermoult (Drach & Tchernigovtzeff, 1967) male crabs ($27.5 \pm 0.7$ mm carapace width) were separated randomly into 3 groups which were kept in glass aquaria containing water of different salinities known to be hypo-, iso- and hyperconcentrated to the internal medium: 12, 34 and 44 g/l. Water was prepared with artificial sea salts (H. W. Marinemix, Wimex R, Germany) added to distilled water. During the 10 d acclimatisation period, the animals were fed twice a week (except for the 48 h before the experiments) and water was changed in intervening days. Prior to dissection, animals were killed by destroying the ventral ganglion by inserting a needle through the ventral side of the body wall.

### *Morphological analysis*

Anterior and posterior gills (numbers 3 and 8) were dissected from crabs acclimatised to different salinities and fixed for 22 to 24 h at 4 °C in Lison-Vokaer fixative, consisting of 10% neutral formalin, 85% ethanol saturated with picric acid and 5% acetic acid, according to Burck (1969). Subsequently, they were dehydrated in absolute ethanol and embedded in paraplast via benzol. Longitudinal thick sections (5–7 μm) were stained with haematoxylin-eosin, Masson's trichrome or by the PAS (periodic acid-Schiff) technique.

### *Morphometric analysis*

All the gills (1–8) were dissected from 6 crabs (2 for each salinity) and fixed in Bouin's solution for 18 h. They were then dehydrated in a graded ethanol series, and finally embedded in paraplast via butyl alcohol. Longitudinal thick sections (5–7 μm) were obtained from dorsal, medial and ventral regions of each gill and stained with haematoxylin and eosin. Both epithelial thickness and cuticle thickness were measured separately using a light microscope with an ocular micrometer at 3 different locations on 3 lamellae of each studied section (1 from each zone). The epithelium and cuticle from the marginal channel were also measured but were not included in the averages.

For statistical analysis, gills were grouped into 3 categories according to a previous description by Luquet et al. (1999): anterior (1, 2, 3), medial (4, 5), and posterior (6, 7, 8). The mean epithelium thickness of the different gills of each category was averaged to obtain a mean value. Repeated measures 2-way ANOVA followed by simple effects tests were performed taking salinity as a fixed factor and gill category as the repeated measures factor. Differences were considered significant where $P < 0.05$.

### *Immunohistochemical identification of cell proliferation*

In order to label proliferating cells, a single dose (1 ml/100 g body weight) of 5-bromo-2′-deoxyuridine (BrdU, Amersham Pharmacia Biotech., Bucks., UK) was given to 6 crabs acclimatised to seawater and 2 crabs acclimatised to 12 g/l salinity, in the prebranchial sinuses. Two seawater acclimatised individuals were then transferred to each of 12, 34 or 44 g/l salinity, while animals acclimatised to 12 g/l were transferred to 44 g/l. After a transfer for 48 h, gills 7 and 8 and hepatopancreas (control tissue) were dissected and fixed as described in the section on Morphological analysis. Sections 5–7 μm were rinsed in 2 5-min phosphate buffered saline (PBS) changes. Endogenous peroxidases were blocked in a freshly made solution of 5% $H_2O_2$ in PBS for 5 min. Tissue sections were treated for 20 min with blocking serum to avoid nonspecific binding, washed in PBS, and then incubated with the primary antibody (purified mouse anti-BrdU monoclonal antibody, Amersham) overnight at 4 °C. Finally, the slides were treated with the Vectastain Elite Kit for mouse antibodies (Vector Labs, Burlingame, CA). The BrdU immunoreactivity was revealed with 0.04% $H_2O_2$ and 0.03% 3,3′-diaminobenzidine solution in 0.1 M Tris-HCl buffer, pH 7.6. The slides were washed in distilled water and in some cases counterstained with eosin. Untreated slides were stained with Masson's trichrome.

The numbers of BrdU-labelled and haematoxylin stained nuclei (total number of nuclei) were counted. The labelling index, L.I. (%) or frequency of labelled nuclei was calculated by the formula: L.I. (%) = number of labelled nuclei/total number of nuclei $\times 100$.

### *Silver nitrate impregnation technique*

Gills 3, 5 and 8 were dissected from 4 crabs of each salinity. Four to 6 lamellae were taken from each gill and treated by the $AgNO_3$ impregnation technique (Koch, 1934) as modified by Kikuchi & Matsumasa (1993). This technique reveals chloride permeable tissue as dark areas due to precipitation of AgCl and subsequent reduction with photographic developer. The proportion of dark area was measured with a Leica Quantimet 520 image analyser attached to a light microscope.

## RESULTS

*C. granulatus* possess 8 pairs of phyllobranchiate gills. The gill lamellae are lined by a single epithelial layer covered by a thin cuticle which defines the diffusion barrier between haemolymph and external medium. The lamellae are expanded laterally in the marginal channel (Fig. 1*b*). Within each lamella the haemolymphatic space is divided into 2 thin sheets by a PAS-positive connective tissue septum (Fig. 1*a*, *b*). There are 2 types of gill epithelium differing histologically and functionally. The gill epithelium in anterior (1–3) gills is thin (e.g. 1.95 μm in gill 1 from 12 g/l salinity crabs), formed by respiratory cells with flattened nuclei and homogeneous cytoplasm. The minimum thickness measured is 0.80 μm. Pillar cells are epithelial with a columnar appearance, 2 or more of these cells joining opposite sides of the lamellae. In anterior gills the epithelium and cuticle thickness from crabs acclimatised to 12, 34 and 44 g/l range from 1.27 to 2.46 μm and 0.78 to 1.79 μm, respectively. No variation in epithelium thickness is seen between dorsal, medial and ventral regions (Table 1), nor between anterior gills from different salinities ($P > 0.05$; Fig. 4).

Gills 4 and 5 show a low cuboid epithelium in some parts of the dorsal and medial regions and thin epithelium in the ventral region, similar to that in anterior gills. At 12 g/l salinity the thickness of the epithelium and cuticle ranges between 2.39 to 4.64 μm and 0.87 to 1.22 μm, respectively.

Posterior gills (6–8) show a cuboid epithelium (e.g. 5.49 to 7.15 μm thick at 12 g/l salinity), with a cuticle thickness similar to anterior ones. The epithelium thickness changes from dorsal to ventral regions, being thicker in the former (Table 1). Cuticle remains constant throughout the gill. The thick cells (ionocytes) are found in dorsal and medial regions of the lamellae, as demonstrated by dark areas with $AgNO_3$ impregnation (Fig. 2). The percentage dark area increases in gill 8 lamellae at low and high salinities compared with seawater (Fig. 3). Ionocytes possess spherical to ovoid nuclei. Pillar cells are hour-glass shaped with ovoid to triangular-shaped nuclei and

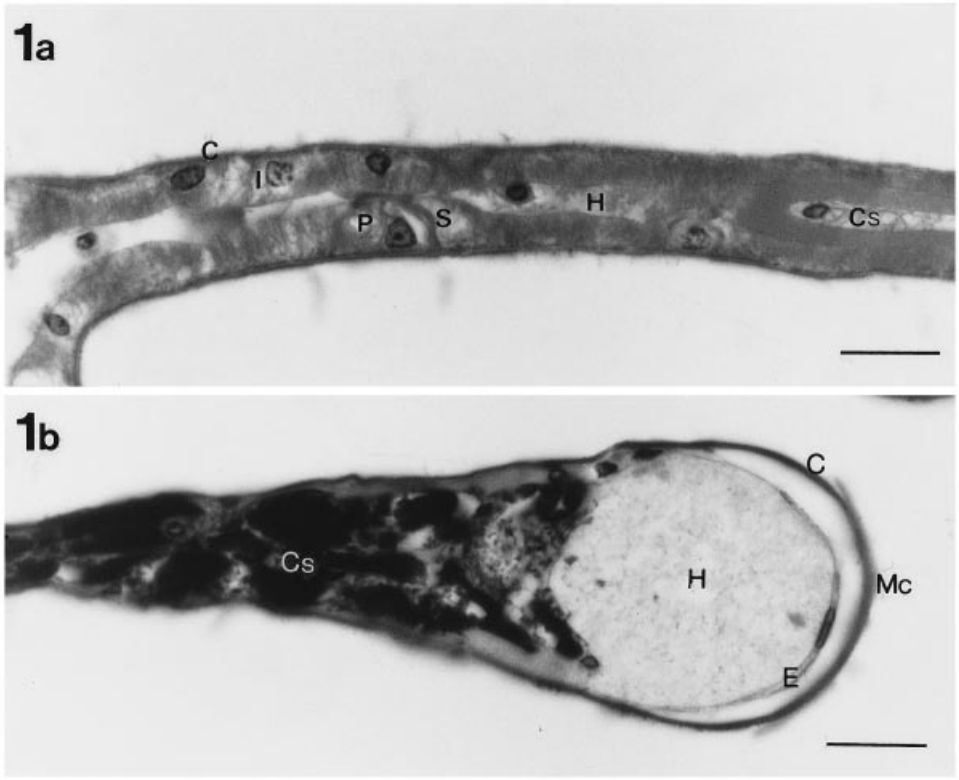


Fig. 1. Longitudinal section of a lamella from gill 8 of a crab acclimatised to 12 g/l salinity. (*a*) Proximal zone. Ionocytes (I) and pillar cells (P) with striations (S) perpendicular to the cuticle (C). The connective septum (Cs) separates the haemolymph (H) space into 2 thin sheets. (*b*) Distal zone demonstrating the attenuated epithelium (E) lining the marginal channel (Mc). The PAS-positive staining of the connective septum (Cs) indicates storage of neutral glycoconjugates. Bar, 20 μm.

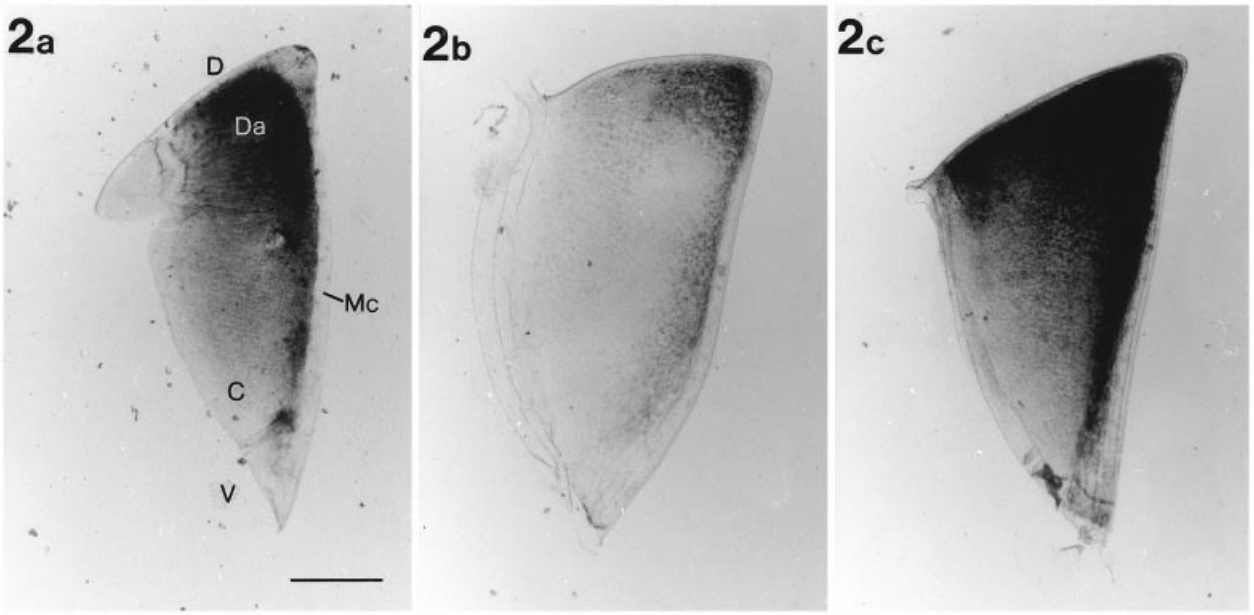


Fig. 2. Whole lamellae preparations from gill 8 treated with $AgNO_3$. Dorsal (D) region showing a dark area (Da), ventral (V) region with clear area (C). Note the difference between salinities. (*a*) 12 g/l. (*b*) 34 g/l. (*c*) 44 g/l. No chloride transporting tissue can be detected in the marginal channel (Mc). Bar, 400 μm.

striations running perpendicular to the cuticle (Fig. 1*a*). In the ventral zones the epithelium tends to be as thin as in the anterior gills.

Results of repeated measures ANOVA show significant differences in epithelial thickness between gill categories and salinities; interaction was also sig-

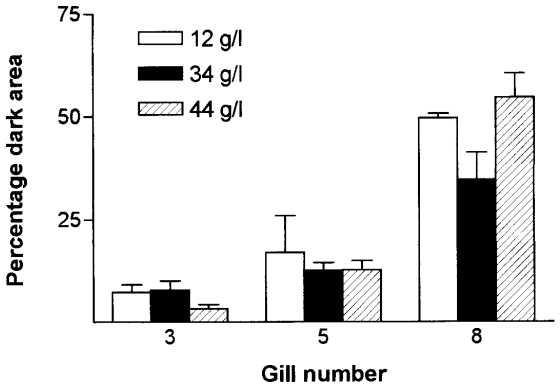


Fig. 3. Percentage dark area (mean ± S.E.) stained with $AgNO_3$ of gills 3, 5 and 8 lamellae from 4 crabs for each salinity.

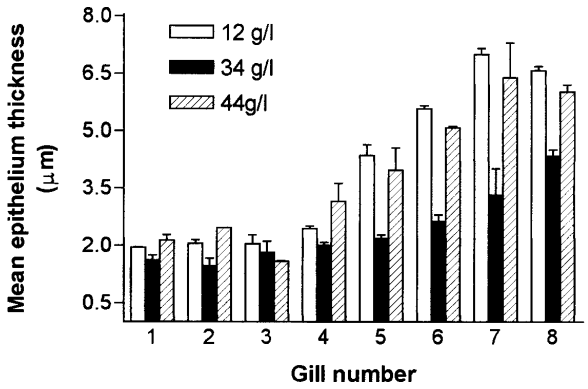


Fig. 4. Mean epithelium thickness ± S.E. (μm) of individual gills of 2 crabs for each salinity.

nificant. Subsequent simple effects indicate that salinity induces significant changes only in the epithelial thickness of posterior (6–8) gills. At low (12 g/l) and high (44 g/l) salinities the gill epithelium is thicker compared with that in seawater (34 g/l) (Fig. 4). Although medial gill epithelial thickness varies with salinity, differences are not statistically significant.

Differences between gill categories are significant at low and high salinities but not in seawater. However, at this salinity posterior gills, especially gill 8, appear thicker than the others, as shown in Figure 4.

The epithelium of the marginal channel of all gills remains fairly constant with external salinity, reaching minimum and maximum values of 0.50 and 2.39 μm in thickness. In this zone the cuticle is slightly thickened (1.0 to 3.5 μm) compared with the rest of the lamella.

The BrdU-immunohistochemical technique for proliferating cells performed in posterior gills reveals a very low labelling index (0.17–1.06 %) both in gills of crabs transferred to a different salinity and in control crabs maintained in seawater. Only a small number of haemolymphatic and epithelial cells with

*Mean epithelium thickness ± standard error (μm) for individual gills of 2 crabs for each salinity*

| Gill | Region | Salinity | | |
|---|---|---|---|---|
| | | 12 g/l | 34 g/l | 44 g/l |
| 1 | Dorsal | 1.82 ± 0.19 | 1.46 ± 0.20 | 2.21 ± 0.18 |
| | Medial | 2.30 ± 0.13 | 1.82 ± 0.17 | 2.13 ± 0.20 |
| | Ventral | 1.59 ± 0.20 | 1.51 ± 0.18 | 1.99 ± 0.22 |
| 2 | Dorsal | 1.7 ± 0.21 | 1.77 ± 0.16 | 2.83 ± 0.35 |
| | Medial | 2.30 ± 0.16 | 1.42 ± 0.14 | 2.39 ± 0.19 |
| | Ventral | 1.95 ± 0.22 | 1.11 ± 0.09 | 2.04 ± 0.27 |
| 3 | Dorsal | 2.39 ± 0.18 | 1.99 ± 0.16 | 1.19 ± 0.18 |
| | Medial | 1.99 ± 0.15 | 1.86 ± 0.17 | 1.51 ± 0.17 |
| | Ventral | 1.45 ± 0.19 | 1.46 ± 0.13 | 1.37 ± 0.20 |
| 4 | Dorsal | 3.28 ± 0.28 | 2.53 ± 0.22 | 3.67 ± 0.51 |
| | Medial | 2.39 ± 0.19 | 1.90 ± 0.23 | 3.37 ± 0.37 |
| | Ventral | 1.55 ± 0.13 | 1.55 ± 0.16 | 1.95 ± 0.29 |
| 5 | Dorsal | 6.0 ± 0.36 | 2.39 ± 0.25 | 5.14 ± 0.50 |
| | Medial | 4.43 ± 0.36 | 2.61 ± 0.26 | 4.34 ± 0.41 |
| | Ventral | 1.90 ± 0.19 | 1.46 ± 0.15 | 2.92 ± 0.43 |
| 6 | Dorsal | 7.09 ± 0.33 | 3.23 ± 0.35 | 5.67 ± 0.64 |
| | Medial | 5.67 ± 0.36 | 2.92 ± 0.23 | 5.18 ± 0.37 |
| | Ventral | 3.52 ± 0.58 | 1.55 ± 0.21 | 4.38 ± 0.38 |
| 7 | Dorsal | 7.60 ± 0.44 | 4.47 ± 0.45 | 6.95 ± 0.65 |
| | Medial | 8.08 ± 0.57 | 3.23 ± 0.29 | 7.00 ± 0.70 |
| | Ventral | 3.99 ± 0.60 | 1.77 ± 0.26 | 5.22 ± 0.69 |
| 8 | Dorsal | 8.10 ± 0.45 | 6.78 ± 0.51 | 7.31 ± 0.68 |
| | Medial | 6.72 ± 0.44 | 3.76 ± 0.31 | 5.88 ± 0.55 |
| | Ventral | 3.77 ± 0.56 | 1.55 ± 0.28 | 4.56 ± 0.55 |

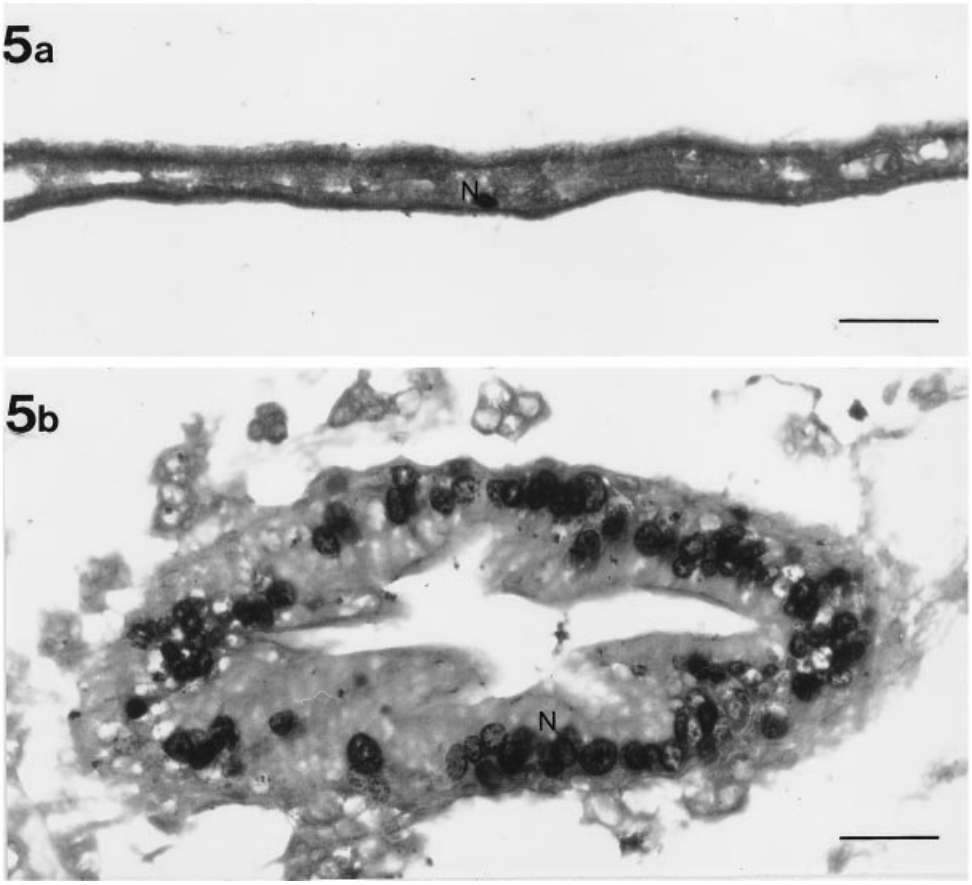


Fig. 5. Longitudinal section of a lamella from gill 8 (*a*) and cross section of hepatopancreas (*b*) from a crab injected with BrdU and transferred from seawater to 12 g/l. In the gill lamella only a single epithelial nucleus (N) is labelled, whereas in hepatopancreas (positive control) numerous nuclei (N) are labelled. Bar, 30 μm.

labelled nuclei can be detected (Fig. 5*a*). Hepatopancreas used as positive control tissue shows a higher L.I., ranging from 28.20 to 56.18 % (Fig. 5*b*).

## DISCUSSION

The morphometric and morphological analyses of anterior (1–3) gills of *Chasmagnathus granulatus* reveal a narrow diffusion distance adequate for gas exchange, as has been reported for the anterior gills of other crabs (Barra et al. 1983; Compère et al. 1989; Goodman & Cavey, 1990; Luquet et al. 1995). As was expected, no reaction with $AgNO_3$ and no variation in epithelial thickness related to external salinity were observed in these gills. The marginal channel of both anterior and posterior gills also shows respiratory characteristics as has previously been reported for this species (Luquet et al. 1999). Both the fact that the least values of epithelial thickness were recorded in this zone, and the lack of variation in cell thickness induced by salinity, suggest an exclusively respiratory function of this part of the lamellae. Since the marginal channel appears to have less resistance for haemolymph flow and it is structurally reinforced, it is expected to be well perfused even when the gills are partially collapsed by loss of branchial water during emersion. Under these conditions the lining of the marginal channels and the neighbouring areas of the lamellae are probably well perfused and exposed to atmospheric air, thus accounting for part of the high metabolic activity reported for this species during emersion (Santos et al. 1987).

*C. granulatus* has been reported as keeping internal ion concentrations similar to those in seawater and to actively hyperregulate at low salinities and hyporegulate at high salinities (Luquet et al. 1992; Mougabure Cueto, 1998). This implies that ions must be actively transported between water and haemolymph either at low or high salinity but not in seawater. The fact that the epithelial thickness and dark area increase in posterior gills after acclimatisation to both high and low salinity, suggests that such gills could be involved both in active ion uptake in diluted seawater and active excretion of ions in hyperconcentrated medium (44 g/l). A great number of studies support the involvement of the posterior

gills of crabs in ion uptake, e.g. Siebers et al. (1982, 1985) have reported a marked increase in $Na^+/K^+$-ATPase activity and a consistent increase of the transepithelial potential differences in the 3 most posterior gills of the hyperregulating crab *Carcinus maenas* after acclimatisation to low salinity. The ultrastructure of the posterior gill epithelium of the same species also changes after transfer from seawater to dilute medium (Compère et al. 1989), an increase in the length of the apical infoldings leading to the development of deep subcuticular infoldings being the most noticeable change reported. Gill ionocytes also appear thicker, probably due to lengthening of the basolateral interdigitations which run parallel to the main cell axis.

The possible role of medial gills remains unclear since we have recorded a tendency to an increase in the epithelial thickness on salinity acclimatisation but this change is not statistically significant. Additionally, there is no noticeable change in silver stained area related to salinity. Preliminary electrophysiological data show almost no active transepithelial potential difference for gills 4 and 5 suggesting that these gills are not involved in active ion transport.

So far there is only a single report providing physiological evidence on the participation of the gill epithelium of a crab (*Ucides cordatus*) in hyporegulation (Martinez et al. 1998). These authors suggested the specialisation of gill 5 in hyperregulation and gill 6 in hyporegulation. On the other hand, we have measured a marked thickening of the epithelium of the 3 most posterior gills and an increase in the putative ion transport area on gill 8 ($AgNO_3$ impregnation technique) parallel to the increase in ionic concentration gradient between haemolymph and water, irrespective of the gradient polarity. The change in the polarity of ion pumping probably occurs through ultrastructural changes. The increase in paracellular permeability, enlargement of subcuticular spaces and organisation of basolateral interdigitations close to the apical membrane in hyporegulating crabs transferred from low to high salinity have already been described for *Uca uruguayensis* and *C. granulatus* (Luquet et al. 1997; Rosa et al. 1999). The latter work shows a temporal correlation between changes in gill ultrastructure and the stabilisation of haemolymph ion concentrations, these representing the most prominent adaptive modifications achieved during the initial 48 h after transfer from low to high salinity.

The dorsal zone of gill 8 of seawater acclimatised crabs seems to be responsible for most of the ion regulation capacities in this medium since the epithelial thickness remains high even in seawater and undergoes little change on acclimatisation. In spite of the fact that in this medium there is nearly no need for ion regulation (Mougabure Cueto, 1998), there is still the possibility of air exposure where ion transport functions are involved in acid-base balance (Burnett & McMahon, 1987; Luquet & Ansaldo, 1997). This thick epithelium also allows *C. granulatus* to cope with abrupt changes in water salinity typical of estuarine coasts. Conversely, gills 6 and 7 and the medial zone of gill 8 suffer noticeable changes in epithelial thickness after acclimatisation to different salinities. This fact might mean that the epithelial linings of these sites are induced by ion gradients to increase their ion transport capacity during long term salinity adaptation.

It has been suggested that gill cells involved in ion uptake from fresh water, in a euryhaline fish, are replaced by salt-excreting cells on transfer to sea water (Wendelaar Bonga & Van Der Meij, 1989; Van Der Heijden et al. 1997). On the other hand, our BrdU-cell proliferation results show that there is almost no cell turnover in the gill epithelium of *C. granulatus* during the initial 48 h of saline adaptation. It could thus be concluded that the increase in epithelial thickness and ion transport area observed in *C. granulatus* after acclimatisation to low and high salinity are caused by thickening of ionocytes already present in the posterior gills of seawater acclimatised crabs. However, another hypothesis involving an acute physiological response (e.g. changes in ATPase activity and number of open ion channels), followed by cell proliferation beginning after 48 h, should not be ruled out and deserves further investigation.

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