GILL MORPHOLOGY OF THE INTERTIDAL ESTUARINE CRAB *CHASMAGNATHUS GRANULATUS* DANA, 1851 (DECAPODA, GRAPSIDAE) IN RELATION TO HABITAT AND RESPIRATORY HABITS

BY

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ABSTRACT

Histological and morphometric analyses were performed on the gills of the semiterrestrial estuarine crab *Chasmagnathus granulatus*. Three different epithelia were recognized: (1) A thin epithelium, 2.42 ± 0.33 μm thick, which lines the whole lamellae in the three anterior gills and was assigned to respiratory functions. (2) A thick cuboidal epithelium, 6-12.5 μm thick, covering part of gills 4 and 5 and the better part of the three most posterior gills. This tissue seems to be involved in ion-regulation, since it is located in the same zones which are darkly stained with silver nitrate and possess large numbers of mitochondria, closely associated to basolateral interdigitations and abundant infoldings of the apical membrane. (3) An attenuated epithelium, 0.5-1.5 μm thick, bordering the marginal channels of all the gills. This tissue is clearly of a respiratory type and probably plays an important role during air breathing when the branchial water stores become reduced by evaporation, and the consequent gill collapse impairs both ventilation and perfusion of the central part of the lamellae.

During exposure to humid air, *C. granulatus* is able to maintain its branchial chambers almost completely filled with water, thus keeping its gills functional in spite of little mechanical support. These adaptations for maintaining gill respiration in air allow *C. granulatus* to sustain high metabolic rates during emergence, with little increase in venous partial pressure of carbon dioxide.

RESUMEN

Se realizaron estudios histológicos y morfométricos de las branquias del cangrejo semiterrestre estuarial *Chasmagnathus granulatus*. Se identificaron tres tipos diferentes de epitelio: (1) Un tejido delgado, de 2.42 ± 0.33 μm de altura, al cual se le atribuyen funciones respiratorias y tapiza toda la superficie de las tres branquias anteriores y parte de las posteriores. (2) Un epitelio cuboide alto de 6-12.5 μm, que cubre parte de las branquias 4 y 5 y la mayor parte de las tres branquias más posteriores. Este tejido puede considerarse ionorregulatorio, ya que se ubica en las mismas áreas que se tiñen intensamente con nitrato de plata y presenta gran cantidad de

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mitocondrias estrechamente ligadas a interdigitaciones de la membrana basolateral y profundos repliegues de la membrana apical. (3) Un epitelio atenuado de 0.5-1.5 μm, que bordea el canal marginal en las laminillas de todas las branquias y tiene un aspecto claramente respiratorio. Este tejido posiblemente cumple un papel importante en la respiración aérea, cuando el volumen de agua retenida en las cámaras branquiales disminuye por evaporación y consecuentemente, las laminillas de las branquias se adhieren entre si, lo cual dificulta la ventilación y la perfusión de su porción central.

Cuando respira aire húmedo *C. granulatus* es capaz de mantener sus cámaras branquiales prácticamente llenas de agua, esto permite mantener funcionales a sus branquias, que son relativamente grandes y carecen de soporte mecánico para la respiración aérea. Estas adaptaciones para mantener la respiración branquial en aire permiten a esta especie mantener una alta tasa metabólica durante la emersión manteniendo bajos niveles de presión de dióxido de carbono.

INTRODUCTION

Brachyuran crabs have evolved from aquatic forms unable to breathe air, to terrestrial forms which cannot survive under water for long periods. Between both groups, the intertidal crabs constitute a large and heterogeneous group of species which represent the transition from water to land. These intertidal crabs can be classified into two main groups:

Low-tide crabs. — These species live near the low-tide mark, being exposed to air only at low tide or when unfavourable conditions force them out of the water. Members of this group are essentially water breathers which display little or no activity during emergence. Their gills are large and not structurally reinforced for maintaining their lamellar spacing in air. The collapse of these structures reduces the surface area available for gas exchange and impairs perfusion, thus reducing both oxygen uptake and carbon dioxide excretion. Consequently, the overall metabolism of these species is reduced during air exposure (De Fur & McMahon, 1984; Burnett & McMahon, 1987). However, certain low-tide species are able to maintain some degree of oxygen uptake by lowering the venous P_{O_2}, thus enhancing the transbranchial P_{O_2} gradient (De Fur, 1988; Henry, 1994).

Amphibious crabs. — This group includes species that spontaneously leave the water and display active metabolism during air exposure (De Fur, 1988). In contrast to aquatic breathers and obligate air breathers, these animals must possess versatile respiratory structures and/or physiological mechanisms to withstand the sudden changes in the respiratory medium caused by active emergence and submergence. According to Henry (1994), some amphibious species have the inner walls of the branchiostegites modified as respiratory organs. These branchiostegal lungs account for most of the oxygen uptake in air. Nevertheless, the gills of these species retain their role in carbon dioxide excretion. Consequently, during air exposure amphibious crabs, even those having branchiostegal

lungs, must keep a water store within their branchial chambers for maintaining the gills moist and also as a temporary sink for carbon dioxide.

Several amphibious grapsid and ocypodid crabs retain large water stores within their branchial chambers. Such water is recirculated over the carapace for aeration and apparently acts as an intermediary medium for both oxygen and carbon dioxide exchange (Hawkins & Jones, 1982; Felgenhauer & Abele, 1983; Santos et al., 1987; Maitland, 1990a, b).

The gill structure of aquatic and low-tide crabs has been extensively studied (Johnson, 1980; Barra, 1983; Taylor & Taylor, 1986; Compere et al., 1989; Lawson et al., 1994). On the other hand, only a few morphometric studies have dealt with the gills of water-recirculating amphibious crabs (Hawkins & Jones, 1982; Santos et al., 1987).

Chasmagnathus granulatus Dana, 1851 is an intertidal grapsid crab widely distributed in salinity-changing habitats along the coasts of Brazil, Uruguay, and Argentina (Boschi, 1964). Crabs of this species spontaneously leave the water and forage on the exposed beach and even in the supra-tidal zone, displaying high locomotor activity. These animals recirculate gill chamber water and are able to sustain a relatively high rate of oxygen uptake in air for several hours (Santos et al., 1987). Upon emergence, *C. granulatus* maintains the same prebranchial P_{O_2} as during water breathing, showing little increase of P_{CO_2} and associated respiratory acidosis. This fall in pH is rapidly compensated by ion transport between the gill chamber water and the hemolymph, across the gills (Luquet & Ansaldo, 1997).

The aim of this work is to describe the gill morphology and ultrastructure of *C. granulatus* in relation to its respiratory habits and capabilities for air breathing and ionic regulation.

MATERIALS AND METHODS

Adult stage C intermoult (Drach & Tchernigovtzeff, 1967) male crabs were collected by hand from a mud-sand flat near Punta Rasa (36°18'S 56°48'W) along the southern edge of the Rio de la Plata estuary, Argentina. In the laboratory the animals were kept in glass aquaria with artificial 12‰ brackish water (H. W. Marinemix, Wimex R salts added to dechlorinated tap water), at 20 ± 2°C, and free access to air breathing.

Light microscopy. — From the right branchial chambers of two crabs all the gills were removed, fixed in a mix of 10% formaldehyde-1% acetic acid,

dehydrated, embedded in paraplast, and cut in 5-7 μm sections, parallel to the gill axis. Finally, the sections were stained with hematoxylin and eosin.

After identification of the various tissues present in each gill, the thickness of the diffusion barrier and each of its parts (epithelium and cuticle) was measured at 3 points, from the base to the edge, of 3 lamellae each, representative of the base, the middle and the tip of the gill. Mean diffusion distance was calculated for each crab as the sum of the mean diffusion distance of each gill, multiplied by the proportional contribution of that gill to the total gill area. The thickness of epithelium and cuticle in the zone of the marginal channel was measured separately and was not included in the mean diffusion barrier calculation.

Transmission electron microscopy. — Gills numbers 3 and 8 were removed from two different crabs, cut into small pieces, and fixed by immersion in 2.5% glutaraldehyde in 0.1 M cacodylate buffer with sucrose (pH 7.4; 650 mOsm l^{-1}). Then, the specimens were immersed in the same fixative for 3 h at 4°C. The tissues were stored overnight in buffer. After being rinsed three times in buffer, they were post-fixed in 1% OsO_4 in the same buffer, at pH 7.2, for 1 h. Later, they were rinsed, dehydrated through an ethanol series, cleared in acetone, and embedded in Spurr's resin. Semi-thin sections (about 1 μm thick) and ultra-thin sections (about 0.07 μm thick) were cut with a Sorvall Porter-Blum ultramicrotome, stained with toluidine blue, and double-stained with uranyl acetate and lead citrate, respectively. Ultra-thin sections were examined in a Siemens ELMISKOP I transmission electron microscope.

Gill surface area. — Gill area was measured on eight adult male crabs following Gray's technique (Gray, 1957). Each crab was blotted dry before weighing (mean weight 12.41±0.78 g). All the gills were dissected from the right branchial chamber and placed on separate slides. Four lamellae were taken every 20 to 25 lamellae along the gill axis, and their surface area was measured by image analysis.

Total gill surface area (GA) was calculated by the following expression:

$$\mathrm{GA} = 4 \times \left(\sum nl \times \mathrm{a} \right)$$

where "nl" represents the number of lamellae counted for each gill, and "a" the average surface area (mm^2) for a single lamella of the same gill. The sum was multiplied by four to take into account both gill chambers and both sides of each lamella.

Ion-regulation area. — All the gills were dissected from the branchial chamber of five crabs, rinsed with distilled water, and processed following the procedure

described by Kikuchi & Matsumasa (1993). Six lamellae were extracted from each gill and treated with 1% $AgNO_3$ in 0.2 M HNO_3. After rinsing with distilled water and 0.2 M HNO_3, the lamellae were fixed in 4% formaldehyde for 1 hour and then treated with photographic developer and fixative. Finally, they were mounted in glycerin and their dark stained areas were measured with an image analyzer.

Branchial chamber water. — Thirty crabs were blotted dry, weighed, submerged in 12‰ water containing 8.5 mCi $^{99m}TcO_4Na$, and then randomly separated in two groups of 15 individuals. After 15 minutes, one group was air exposed in a closed container with high ambient humidity. Then the animals were transferred to individual vessels with 100 ml of distilled water. The other group was directly transferred from the initial water to the individual vessels with distilled water. Both groups were kept in distilled water for 15 min., then samples from each one of these vessels and from the initial 12‰ water were analysed for gamma emission in a Clinigamma 1272 LKB-Wallac (Switzerland). These measurements were made all at the same time, in order to avoid differences due to decay in radioactivity. The water volume retained by each crab was estimated by the following formula:

$$B_w = E_f \times V_f / E_i$$

where B_w is the volume of water contained within the branchial chambers, E_f is the gamma emission of the final solution, V_f is the volume of the final solution and E_i is the gamma emission of the initial 12‰ water.

Water adhered to the carapace was subtracted by repeating the same experimental protocol with two dead crabs with their respiratory openings sealed with acrylic filler. Results from both groups (submerged and emerged) were compared by one-way analysis of variance (Sokal & Rohlf, 1981).

RESULTS

Chasmagnathus granulatus possesses eight pairs of phylobranchiate gills, pairs 1 and 2 being greatly reduced but still functional. The branchial formula of this species is described in table I.

Each gill lamella is a flattened hemocoelic space lined with a simple epithelium covered by cuticle. At the edge of the lamellae the hemocoelic space is expanded forming the marginal channel. A connective tissue septum, often thicker than the epithelial layers, runs through the lamellar hemocoel finely par-

TABLE I
Branchial formula of the crab *Chasmagnathus granulatus* Dana, 1851

	Thoracic appendages		
	1 2 3 (Maxillipeds)	4 (Chelipeds)	5 6 7 8 (Peraeopods)
Podobranch	– 1 1	–	– – – –
Arthrobranch	– – 2	2	– – – –
Pleurobranch	– – –	–	1 1 – –

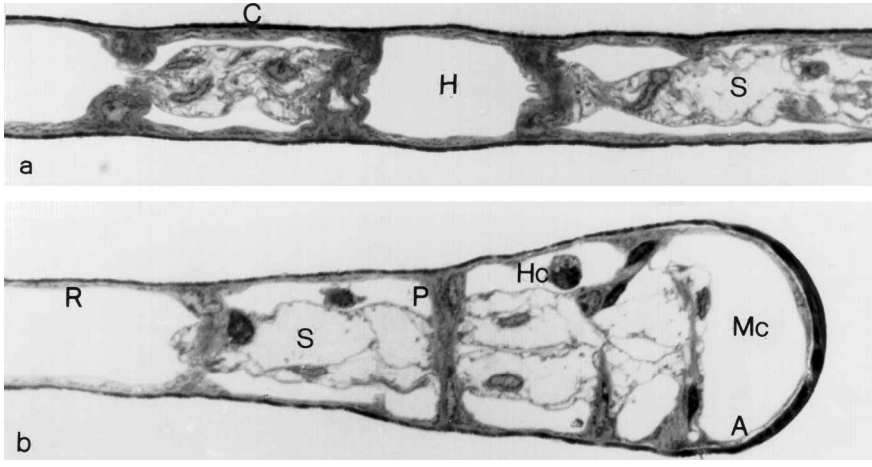


Fig. 1. Longitudinal sections of gill 3 of *Chasmagnathus granulatus* Dana, 1851. a, median zone of the lamellae; b, distal zone: A, attenuated tissue; C, cuticle; H, hemolymph; Hc, hemocyte; Mc, marginal channel; P, pillar cell; R, respiratory tissue; S, connective septum. 630×.

titioning the lamellar circulation. Numerous hemocytes are seen in these lacunar spaces (figs. 1, 3).

Two types of epithelial tissue can be recognized, respiratory and ion-regulatory:

Respiratory epithelium. — The tissue lining the whole surface of the anterior gills and part of the posterior ones principally consists of thin, squamous cells with broad lateral expansions 2.42 ± 0.33 μm thick (fig. 1). This tissue is covered by a 1.32 ± 0.18 μm thick cuticular layer, the cuticle lining the marginal channel was not considered for calculation of means.

At an ultrastructural level, the apical membrane of respiratory cells is deeply infolded in a brush border fashion. Neighbouring cells are joined by long septate desmosomes followed apically by a zonula adherens. Few mitochondria are scattered in the cytoplasm. Basolateral membranes of neighbouring cells show few interdigitations not associated to mitochondria (fig. 2).

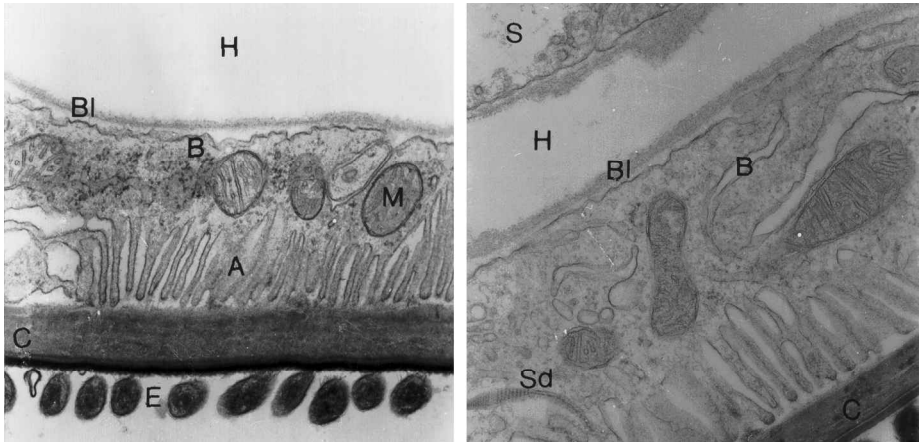


Fig. 2. Electron micrographs from gill 3 of *Chasmagnathus granulatus* Dana, 1851. A, apical membrane; B, basolateral membrane; Bl, basal lamina; C, cuticle; E, epibionts; H, hemolymph; M, mitochondrion; S, connective septum; Sd, septate desmosome. 20 000 ×.

Pillar cells are frequently observed within this epithelium. Two pillar cells from opposite sides abut in the middle of the lamellar hemocoel. These cells possess thin lateral expansions similar to those in respiratory cells and a central elongated part almost obliterated by rows of microtubules oriented perpendicular to the cuticle. Pillar cells are often anchored to the connective septum, providing mechanical support and contributing to the partitioning of the hemocoelic space (fig. 1b).

Ion-regulatory epithelium. — This tissue covers the dorsal portion of the lamellae of the gills 4 to 8. In semi-thin sections, it appears to be constituted of cuboidal shaped cells 6-12.5 μm thick, with spherical nuclei (fig. 3a). The zones of the lamellae lined with thick tissue observed in transversal sections correspond to darkly stained areas as seen in whole lamellae treated with $AgNO_3$ (fig. 4b).

Ultrastructurally, this thick epithelium shows characteristics of an ion-transporting tissue. The apical plasma membrane is greatly infolded, defining irregular subcuticular spaces. Abundant mitochondria, often circular shaped appear associated with the apical infoldings. Basolateral membranes of adjacent cells are intricately interdigitated. Great numbers of mitochondria, bearing numerous cristae, are closely packed between these interdigitations. The apical and perinuclear regions of thick cells show abundant rough endoplasmic reticulum, and vesicles often associated with numerous microtubules (fig. 5). Pillar cells within this tissue are similar to the neighbouring thick cells, but they show a basal cytoplasmic prolongation towards the centre of the lamellae, where they abut with the opposite pillar cell. Typically, conspicuous bundles of microtubules are present in these cells, running from the basal to the cuticular extreme (fig. 3a).

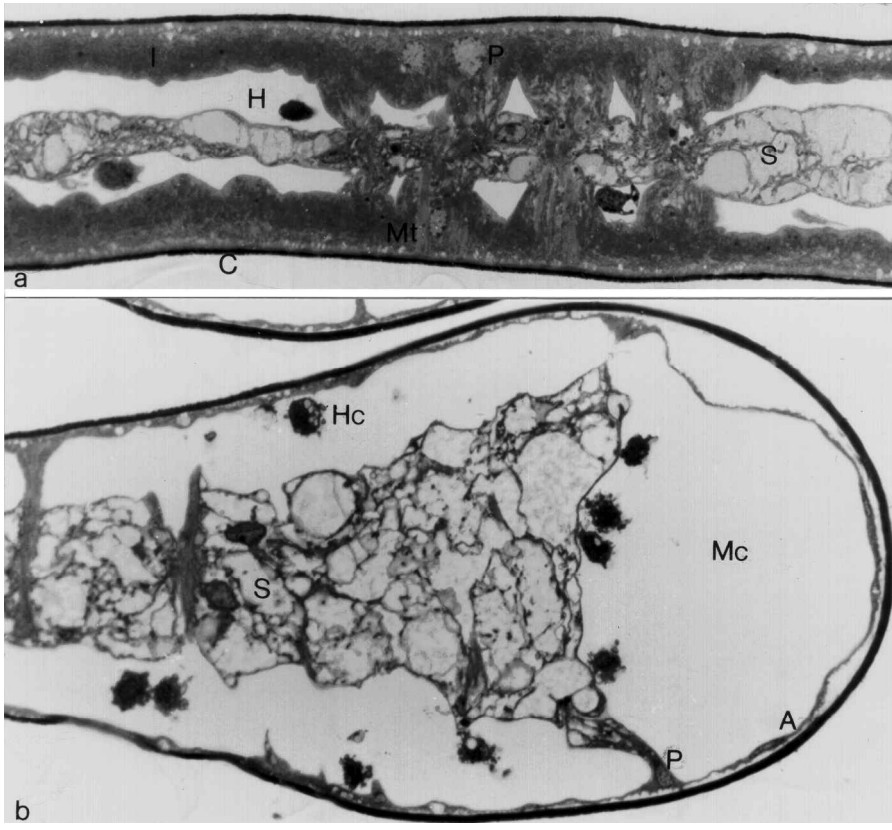


Fig. 3. Longitudinal sections of gill 8 of *Chasmagnathus granulatus* Dana, 1851. a, median zone of the lamellae; b, distal zone: A, attenuated tissue; C, cuticle; H, hemolymph; Hc, hemocyte; I, ion-regulation tissue; Mc, marginal channel; Mt, microtubules; P, pillar cell; S, connective septum. 630×.

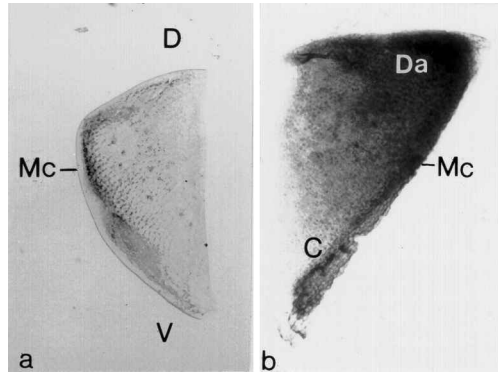


Fig. 4. Whole lamellae preparation treated with $AgNO_3$ of *Chasmagnathus granulatus* Dana, 1851. a, gill 3; b, gill 8: C, clear area; D, dorsal; Da, dark stained area; Mc, marginal channel; V, ventral. 40×.

All the gills, despite their respiratory or ion-transporting characteristics, exhibit marginal channels lined with a very thin epithelium, 0.5-1.0 μm in gill 3 and 0.5-1.5 μm in gill 8. The cuticular layer is slightly thickened in this zone, up to 3 μm in the three most anterior gills and 2.5 μm in the posterior ones. The circulatory space of the marginal channel is often finely partitioned by expansions of the lamellar septum, which are supported by complexly branched pillar cells (figs. 1b, 3b).

Gill surface area. — The regression equation between specific gill area and fresh body weight is:

$$\log GA = 7.26 - 0.357 \times \log W; \quad r^2 = 0.70; \quad P < 0.05$$

where GA is the specific gill surface area in $mm^2\ g^{-1}$ and W is the fresh body weight in g. The specific gill surface area calculated for a 12 g crab is 585.75 $mm^2\ g^{-1}$.

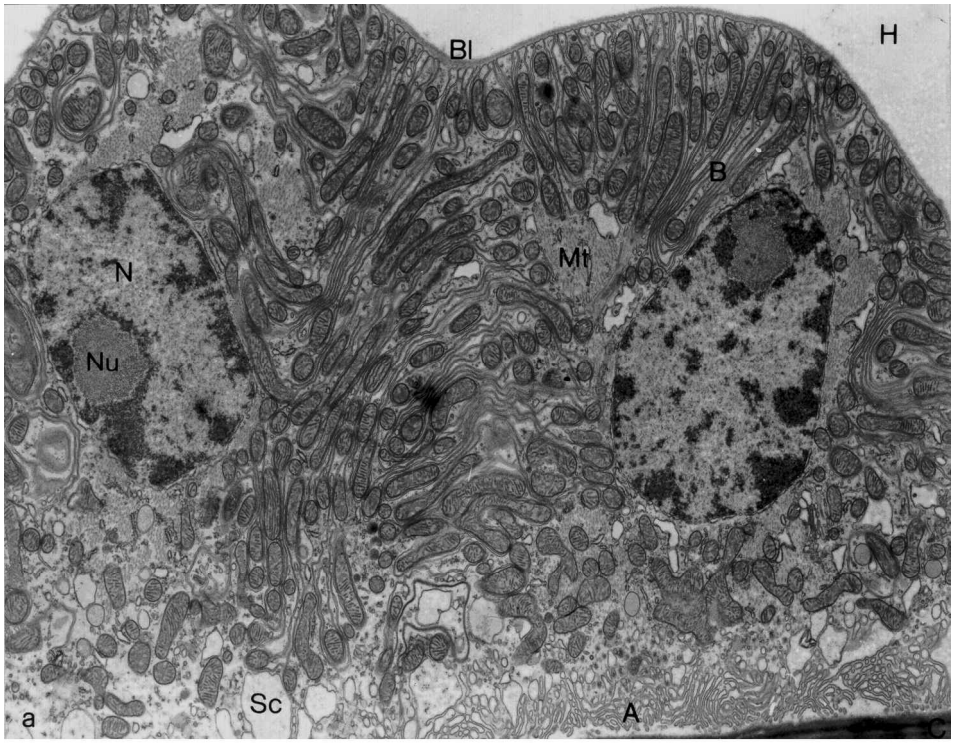
Ion-regulation area. — Treatment with $AgNO_3$ yielded a darkly stained area which accounts for 51% of the total gill area, results for each gill are detailed in table II.

Branchial chamber water. — The maximum volume of water contained within the branchial chambers (measured in submerged crabs) is 83.21 ± 12.63 $\mu l\ g^{-1}$. After 1 h of air exposure, there is a slight and not significant decrease in this volume to 73.87 ± 6.67 $\mu l\ g^{-1}$ ($P > 0.05$). The total branchial water volume calculated for a 12 g crab submerged and emerged is 1 ml and 0.89 ml, respectively.

TABLE II

Respiratory and ion-regulation areas measured in gill lamellae of the crab *Chasmagnathus granulatus* Dana, 1851, treated with $AgNO_3$

Gill	Total area (mm^2)	Dark area (mm^2)	Respiratory area (mm^2)
1	108.81 ± 15	0.00 (0)	108.81 (100)
2	75.75 ± 11	0.00 (0)	75.75 (100)
3	648.96 ± 77	0.00 (0)	648.96 (100)
4	1511.17 ± 132	468.46 (31)	1042.71 (69)
5	2180.76 ± 311	1024.95 (47)	1155.81 (53)
6	1031.55 ± 127	804.61 (78)	226.94 (22)
7	852.83 ± 98	699.32 (82)	153.51 (18)
8	642.59 ± 82	597.61 (93)	44.98 (7)
Total	7052.43 ± 733	3595.66 (51)	3456.77 (49)
N	8	5	5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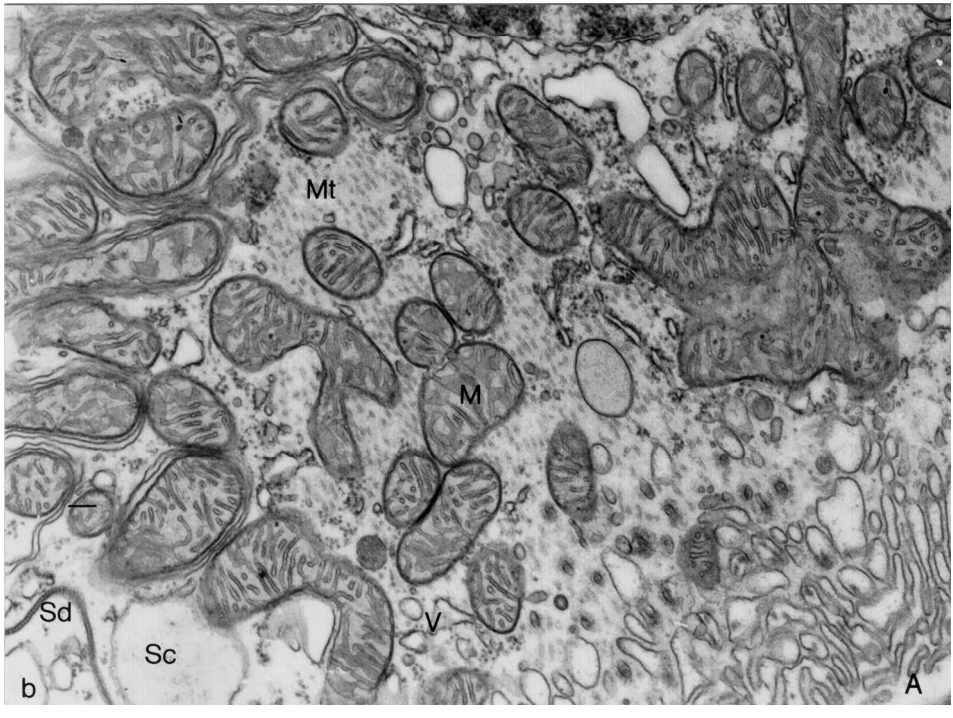
Mt
M
Sd
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DISCUSSION

The general gill structure of *Chasmagnathus granulatus* resembles those in aquatic and low-tide crabs (Johnson, 1980; Taylor & Taylor, 1986). The fact that *C. granulatus* possesses relatively larger gills lined with thin cuticle, in comparison with active air breathers such as *Ocypode quadrata* (Fabricius, 1787) (as *O. albicans* (Bosc, 1802)) and *Uca* species (Gray, 1957; Rabalais & Cameron, 1985), suggests that the gills of this species would collapse if the branchial water stores were reduced. The slight cuticular thickening observed over the marginal channels of the gills of *C. granulatus* does not seem to be enough to bring support to the whole organ, as it does in semiterrestrial and terrestrial crabs from the families Gecarcinidae and Ocypodidae (cf. Cameron, 1981; Al-Wassia et al., 1989; Luquet et al., 1995). These marginal cuticular reinforcements in *C. granulatus* are more likely involved in maintaining hemolymph flow through the marginal channel during emergence, ensuring the perfusion of its own epithelium and the adjacent tissues.

Accordingly, the tissue lining the marginal channel is markedly attenuated, about 0.5-1.5 μm thick, in all the gills. A similar attenuated epithelium thought to play respiratory functions was reported by Taylor & Taylor (1986) for the marginal channel of the gills of *Carcinus maenas* (Linnaeus, 1758), which is another air breather possessing large gills. In contrast, the marginal channel in the reduced gills of *Uca uruguayensis* (Nobili, 1901) is lined with an intermediate tissue and a thick cuticle (Luquet et al., 1995), suggesting that in species which do not keep large water volumes within their branchial chambers, the main functions of the marginal channel are mechanical support of lamellae and hemolymph circulation but not gas exchange.

The connective septum of *C. granulatus* gills becomes expanded into the marginal channel, defining narrow circulatory pathways just beneath the attenuated epithelium. Pillar cells are more frequent near the marginal channels and their branches attach to the connective septum, apparently protecting the circulatory structure from changes in perfusion pressure.

On the other hand, the fact that *C. granulatus* stores a large water volume within its branchial chambers and recirculates it over the carapace suggests that the gills are generally well supplied with ventilated water. However, whilst water is recirculated, some air may pass through the branchial chambers, ventilating

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Fig. 5. Electron micrographs from gill 8 of *Chasmagnathus granulatus* Dana, 1851. a, general view of ionocytes; b, apical detail: A, apical membrane; B, basolateral membrane; Bl, basal lamina; C, cuticle; H, hemolymph; M, mitochondrion; Mt, microtubules; N, nucleous; Nu, nucleolus; Sc, subcuticular space; Sd, septate desmosome; V, vesicles. a, 12 000 ×, b, 40 000×.

the inner branchiostegal integument and also possibly the dorsal lamellar margins of the gills. Thus, the high metabolic rate sustained by emerged *C. granulatus* (cf. Dezi et al., 1987; Santos et al., 1987) is accounted for by aquatic exchange through the gills, ventilated by water recirculation, plus direct exchange with air through the lamellar margins and branchiostegites. Physiological experiments indicate that in this species branchial water is especially involved in CO_2 excretion, whereas O_2 is efficiently taken up from the air (Halperin, 1997).

The efficiency of this respiratory system can be measured by an index defined by the ratio between the rate of oxygen consumption and gill area (V_{O_2}/GA), which reflects the apparent oxygen flux by unit of gill area. Considering the aerial V_{O_2} for a 12 g crab as 0.71 μmol min.$^{-1}$ (Dezi et al., 1987), and the gill area calculated from the regression equation reported here for a similar sized animal, 7029 mm^2, the V_{O_2}/GA index equals 1.00×10^{-4} μmol min.$^{-1}$ mm^{-2}. This value lies near the lowest limit for air breathers reported by Luquet et al. (1995) and is two- to fourfold higher than those reported in the same paper for aquatic breathers. On the other hand, the relatively large gills of *C. granulatus* enable this species to maintain high rates of oxygen uptake from the air with little increase in venous P_{CO_2} in comparison with semiterrestrial and terrestrial crabs, which possess more reduced gills (Al-Wassia et al., 1989; Farrelly & Greenaway, 1994).

The branchial chamber volume measured in this paper is similar to those in amphibious and terrestrial crabs possessing smooth lungs (Maitland, 1990a). Thus, when the branchial chamber is partially emptied, the aerial gas exchange is allowed to occur over a large surface area. However, if ambient humidity is high or the crab is recently emerged, the great volume of water retained favours gill exchange and limits aerial oxygen uptake to the air volume which replaces circulating water.

The dark zones which account for 51% of the total gill area correspond to thick tissue with abundant mitochondria closely packed between infoldings of the basolateral membrane. These associations were termed by Copeland (1968) as "mitochondrial pumps". These kind of cells have been regarded as playing an ion-transporting role, packed mitochondria being responsible for supplying energy to basolaterally placed Na^+K^+ ATPase (Gilles & Péqueux, 1986; Taylor & Taylor, 1992; Lucu, 1993). Apical mitochondria associated with the infoldings of the apical membrane seem to be involved in supplying energy to another active transporter such as the proton pump (H^+ V-ATPase), which has recently been reported to be present in crustacean gills (Krippeit-Drews et al., 1989; Onken, 1996). The presence of vesicles associated with microtubules near the apical membrane suggests an endocytic/exocytic activity as was described for

mitochondria-rich cells of vertebrate tissues containing H^+ V-ATPase (Brown et al., 1997). In euryhaline crabs like *C. granulatus* this enzyme is possibly involved in energizing ion transport through the apical membrane from a dilute medium (Wright, 1991) or under acidic conditions. Since this species has been reported as compensating the respiratory acidosis caused by emergence through ion uptake from the branchial chamber water (Luquet & Ansaldo, 1997), the presence and complexity of the gill ionocytes appears to be related to the capability of *C. granulatus* to invade low and high salinity media and also to exploit supralittoral resources. The large branchial water stores allow this species to maintain CO_2 excretion through the gills during emergence, with lower increase of hemolymph CO_2 than most amphibious and terrestrial crabs (Farrelly & Greenaway, 1994). These water stores also provide the ion source and proton sink needed for acid-base compensation based on ion transport.

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