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Original Article

Histological localization of the mucus-secreting cell, sacciform cell, and pigment cell in the integument system of the grunting toadfish, *Allenbatrachus grunniens* (Linnaeus, 1758)

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Abstract

With limited observations, one of the major remaining questions in toadfish biology is how the integument structure helps them cope with a harsh estuarine environment. In this study, we attempted to observe the structure and histological characteristics of the integument system (classified into seven areas) in the grunting toadfish, *Allenbatrachus grunniens*, an economically important estuarine fish. All samples were collected from the estuarine of Pranburi River, Thailand. The skin of *A. grunniens* consists of two layers (epidermis and dermis). The epidermis contains several types of cells, including the mucus-secreting cell, sacciform cell, pigment cell, and Malpighian cell. Based on the morphometric data analysis, the thickest epidermis was observed near the frontal part (area 1), while the thinnest was seen in the middle part between pelvic fins (area 5). The high density of mucus-secreting cells was demonstrated by the periodic acid-Schiff method in area 6 (posterior part of the pectoral fin), area 1 (upper jaw), and area 7 (caudal fin), whilst area 2 (frontal part) and area 4 (lower jaw) displayed a low density of mucus-secreting cells. Interestingly, toadfish samples from the high salinity environment showed significantly higher numbers of mucus-secreting cells than those from the low salinity environment in the integument system areas 3, 4, 5, and 7 ($P < 0.05$), which is the first report from this toadfish. This change might be responsible to an adaptation to changes in salinity, representing an important ability to cope with the estuarine environment.

Keywords: dermis, histology, integument, mucus-secreting cell, skin, Thailand, toadfish

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1. Introduction

It is well known that the estuarine area is a vital component of the transition zone between river and marine environments. Large fluctuations of the salinity and oxygen levels are characteristic of estuarine areas depending on the locality, tidal range, freshwater flow volumes, etc. This constitutes a major problem for estuarine animals, which have to adapt to the fluctuating environment by changing the system, organ, and cellular physiology. In this regard, the integumentary system plays an important role in the adaptation and survival of estuarine animals because it serves as the first barrier against environmental changes (Bonga & van Der Meij, 1981; Laurent, 1984). However, there is a remarkable lack of data on the teleost integumentary system from the viewpoint of osmoregulation and hypoxia tolerance.

The potential role of the mucus cell in osmoregulation has been suggested in the literature (Powell, Speare, & Wright, 1994; Scott, 1989). For example, Shephard (1994) documented different functions of fish mucus, including osmotic and ionic regulations. Similarly, Yuge *et al.* (2003) reported that mucus secretion is increased in response to increasing luminal osmolality, suggesting the role of mucus-secreting cells in water regulation and salt absorption. The adaptation to hypoxia is also regulated, at least partly, by the integumentary system in teleosts (Bond, 1996; Graham, 1997). The percentage of oxygen exchanged through the integument systems likely rises to about 30 percent when under low oxygen levels (Bond, 1996; Feder & Burggren, 1985; Graham, 1997).

Although the histological structures of the integument system have been reported for several fish species, such as *Morone saxatilis* and *Gnathonemus petersii*, *Danio rerio* (Groman, 1982), and *Pelvicachromis pulcher* (Genten, Terwinghe, & Danguy, 2009), little is known about the integument structure of estuarine fish. One of the few reports was about the integument system of *Callionymus schaapii*, in which the detailed structure of outer epidermis and underlying dermis was demonstrated (Senarat *et al.*, 2020). In most fish species, the microanatomical structure of the body wall is comprised of six layers, including the cuticle, epidermis, basement membrane, dermis, and hypodermis (Roberts, 2012), and these layers are the first protective barrier against environmental changes (Genten *et al.*, 2009; Groman, 1982).

The grunting toadfish, *Allenbatrachus grunniens* (Linnaus 1758), is one of the most economically important demersal fish living in the estuarine environment. It commonly inhabits the muddy bottoms of coastal waters and estuarine areas throughout Indo-West Pacific waters, particularly in Thailand (Keegan, Toshioka, & Suzuki, 1968). Due to limited observations, how the integument structure helps *A. grunniens* cope with the harsh estuarine environment remains unclear. This study attempted to develop an initial understanding of the structure of the integument system in *A. grunniens* by comparing several locations of its body using histological and histochemical techniques with morphometric analysis. Also, we compared the number of mucus-secreting cells in fish obtained from different salinity levels, which may help to reveal the mechanism by which *A. grunniens* can live in the estuarine environment.

2. Materials and Methods

2.1 Fish collection and study area

Fixed healthy specimens of *A. grunniens* (n = 6 with 18.19 ± 1.93 cm in total length) were donated and therefore considered to be a voucher specimen from Mitparian (2018), and were kept at the Fish Biology and Aquatic Health Assessment (FBA-LAB), Department of Marine Science, Faculty of Science, Chulalongkorn University, Thailand. These fish were collected at the Pranburi River estuary, Thailand (N12º 24.267' E099º 58.425') at two different time points. One group (n = 3 individual samples) was collected under the high salinity (24.0 PSU) in December 2016. In contrast, the other (n = 3 individual samples) group was collected under low salinity conditions (11.13 PSU) in January 2017 (Mitparian, 2018). The experimental protocol was approved by the Animal Care and Use Committee of the Faculty of Science in accordance with the guide for the care and use of laboratory animals prepared by Chulalongkorn University (Protocol Review No. 1723004).

2.2 Light microscopic observation

Seven distinct areas were chosen on the *A. grunniens* integument system (Figure 1): area 1 = upper jaw, area 2 = frontal part, area 3 = lateral part of the middle body, area 4 = lower jaw, area 5 = chin, area 6 = posterior part of the pectoral fin and area 7 = caudal part. Tissue samples of about 5 × 5 mm^2 were dissected from each area, and all samples were processed using standard histological techniques according to Presnell & Schreibman (1997) and Suvarna, Layton & Bancroft (2013). Tissue blocks were serially sectioned at 4 µm thickness and stained with Harris's hematoxylin and eosin (H&E). Sequential sections were histochemically analyzed after histological observation using periodic acid-Schiff (PAS) to detect the glycoprotein, alcian blue (AB) to detect the mucopolysaccharide, and Masson's trichrome (MT) to observe the fiber and connective tissue. Moreover, frozen tissues were also cut to 20 µm in thick and stained with oil red O (ORO) to detect lipid deposition (Culling, 1974). A light microscope was used to identify and compare the histological structures, and photographs were taken with LEICA DM750-Ua microscope equipment.

2.3 Morphometric analysis and estimate of cell density

The thickness of two integument layers, epidermis and dermis, was recorded in all areas. Data are shown as the mean and standard deviation (SE). The density of mucus-secreting cells, sacciform cells, and pigment cells in the epidermis were examined in the histological sections. Three representative slides were selected from each area, and each slide was observed at ten randomized areas to count the number of cells at 40× under a light microscope. The density of sacciform cells and pigment cells represented as the cell density index, including zero cell = 0 score, 1-2 cell = 1 score, 3-4 cells = 2 scores, 5-7 cells = 3 score, 8-10 cells = 4 scores and 10+ cells = 5 scores, following adapted guideline of

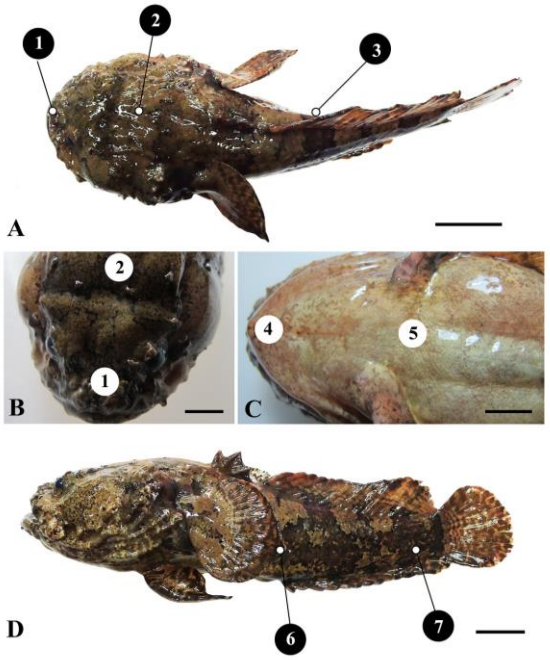

Figure 1. External morphology of *Allenbatrachus grunniens* showing the histologically observed areas marked 1-7. (A) The overall morphology includes area 1 = upper jaw, area 2 = frontal part, and area 3 = lateral part of middle body. (B) The head part containing areas 1 and 2. (C) The dorsal view shows area 4 = lower jaw and area 5 = chin. (D) Lateral view containing area 6 = posterior part of pectoral fin and area 7 = caudal part.

Solanki & Benjamin (1982). In particular, the density of the mucus-secreting cells was numerically analyzed in samples from different salinities.

2.4 Statistical analysis

Normality of distribution was tested using the Kolmogorov-Smirnov Test (K-S Test). The statistical significance of the morphometric analysis of integument and mucous-secreting cell count was assessed using a paired-sample t-test. Statistical significance was called for $P < 0.05$ by using GraphPad Prism for Windows.

3. Results and Discussion

3.1 Histological structural evidence of integument

The present study demonstrated, for the first time, the structure of the integument system of the grunting toadfish *A. grunniens.* The integument system consisted of two layers: epidermis and dermis (Figure 2A). Similar observations have been made for relatively few species: *Gnathonemus petersi*, *Astronotus ocellatus* (Mittal & Munshi, 1970), and *Bagarius bagarius* (Harder, 1975). Other fishes' integument system consists of three principal layers: epidermis, dermis, and hypodermis (Mittal, Whitear & Agarwal, 1980; Park & Kim, 1999, 2000; Whitear, 1986).

The microscopic examination demonstrated that the epidermis is a complex tissue, which consists of the outermost

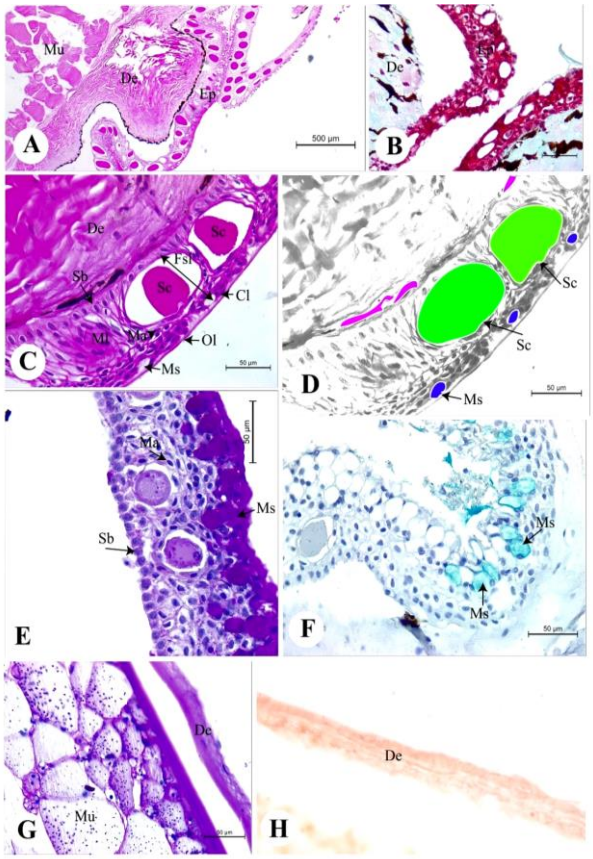

Figure 2. Light photomicrograph of the integument system of *Allenbatrachus grunniens* consisting of two layers [epidermis (Ep) and dermis (De)] (A-B). Several cells, including mucus-secreting cells (Ms), sacciform cells (Sc) and Malpighian cells (Ma), were observed in the epidermis (C-F). The muscular element (Mu) was identified below the dermis (G). The positive reaction of oil red O of the dermis was detected as the orange color (H). Note: Cl = cuticular sub-layers, Fsl = fusiform sub-layer, MI = middle layer, Ol = outermost layer, Pc = pigment cells, Sc = sacciform cells (green color), Sb = stratum basal. Staining method: A, C = Harris's hematoxylin and eosin (H&E); B = Masson's trichrome (MT), E, G = periodic acid-Schiff (PAS); F = alcian blue (AB); H = oil red O (ORO)

layer, the middle layer, and the stratum basal layer (Figure 2C). The outermost layer was lined up apically in the epidermis and consisted of numerous polygonal cells. These cells had an irregular, flat shape and formed approximately 2–6 layers (Figure 2C). The middle layer is situated under the outermost layer. This layer is further divided into the fusiform and cuticular sub-layers (Figure 2C). The fusiform was a stratified layer encompassing three types of cells: mucus-secreting (or goblet cell), sacciform (or sacciform granulated cell/club cell), and Malpighian cells (or epidermal cell) - identified depending upon the shape and histological details under the light microscopy level (Figures 2C-2F). The mucus-secreting cells of the oval and spherical shape were 15 µm in height (average; range 15–24 µm) and observed as vacuoles by the H&E method (Figure 2C). The mucus-secreting cells were more clearly observed by applying the PAS and AB

techniques (Figures 2E, 2F), which stain glycoproteins and mucopolysaccharide contents. The cell shape and histochemical characteristics of the mucus-secreting cell are similar to those in *Acipenser gueldenstaedii* and *Pangio kuhlii* (Genten *et al.*, 2009) and *Callionymus schaapii* (Senarat *et al.*, 2020). The role of mucus-secreting cells is the regulated moisturization of body surfaces, stress protection, predator avoidance (Genten *et al.*, 2009), and osmotic and ionic regulation (Shephard, 1994). The sacciform cell was the largest cell of a pear shape, which contained large vesicles (H&E and PAS stains) (Figures 2C, 2E). This feature resembles other fish (Agrawal & Mittal, 1992; Whitear, 1986; Zaccone, Kapoor, Fasulo & Ainis, 2001). Agrawal & Mittal (1992) reported that the sacciform cell secretes pheromones. It is also speculated that the sacciform cells produce noxious substances to protect against pathogenic microorganisms and alarming substances (Mittal, Whitear & Bullock, 1981; Roberts, 2012). The last cell type, the Malpighian cell, is an elongated cell located near the cuticle layer (Figure 2E). The thin layer of the stratum basal is accompanied by the low, simple cuboidal epithelium (Figure 2C), which was situated on the basement membrane. The Malpighian cell contained an oval basophilic nucleus surrounded by the eosinophilic cytoplasm (Figure 2C).

The dermis was detected below the basement membrane. It contained bundles of loose connective tissue parallel to the skin surface (Figure 2A). This layer was a deep green in the MT method (Figure 2B) and located on the muscular element (Figure 2G). Interestingly, the pigment cell (or chromatophores) was found in the dermis of this species (Figures 2B-2D). This finding was consistent with a previous report by Gona (1979), who found the pigment cells in the stratum spongiosum of *Pangio kuhlii* and *Astronotus ocellatus*. On the other hand, the pigment cells were found throughout dermal and epidermal layers in *Astatotilapia lapiaburtoni* (Gona, 1979), some fishes (Elliott, 2010). The pigment cells are crucial for fish coloration (Takeuchi, 1976).

Lipids were only detected in the dermis with oil-red O staining (Figure 2H). While the subcutaneous lipid is well-known, there have been few observations of the presence of the lipid in the dermis.

3.2 Morphometric analysis and semiquantitative scoring for cell density

According to the morphometric data, the average thickness of the integument system was different in the seven selected areas (Figures 5A-5G). In all areas tested, the dermis was significantly thicker ($P < 0.05$) than the epidermis, similarly as seen in *Eremophilus mutisii* (Bonilla Lizarazo *et al.*, 2008) and *Polyodon spathula* (Mester & Zarnescu, 2000). This difference may have some functional implications, which need further studies.

The detailed cell characteristics in each area are shown in Figures 3-4 and 6A-5G. The high density was in areas 6, 1, and 7 and the low in areas 2 and 4 existed. The high density of the mucus-secreting cells in the upper jaw, posterior part of the pectoral fin, and caudal part might be related to the lubrication and protection of fish against abrasive injuries during search for food on the bottom.

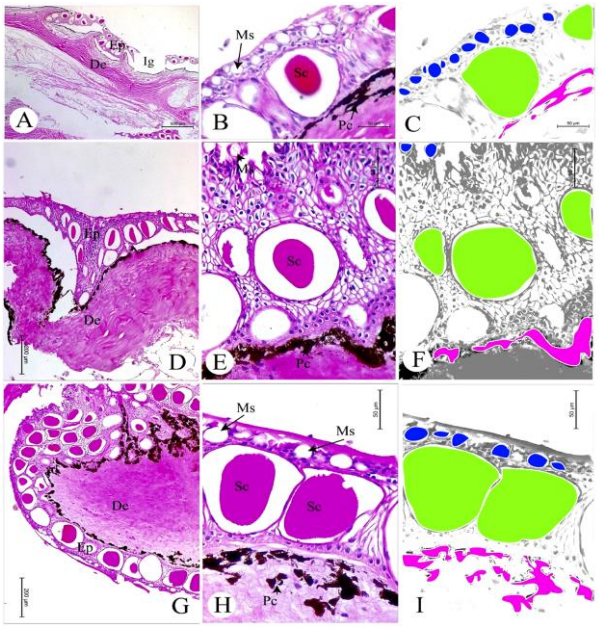

Figure 3. Representative light photomicrograph and schematic diagram showing the integument areas 1 (A-C), 2 (D-F), and 3 (G-I) of *Allenbatrachus grunniens*. Note: Ep = epidermis, De = dermis, Ms = mucus-secreting cells (blue color), Sc = sacciform cells (green color), Pc = pigment cells (pink color). Staining method: A, B, D, E, G, H = Harris's hematoxylin and eosin (H&E)

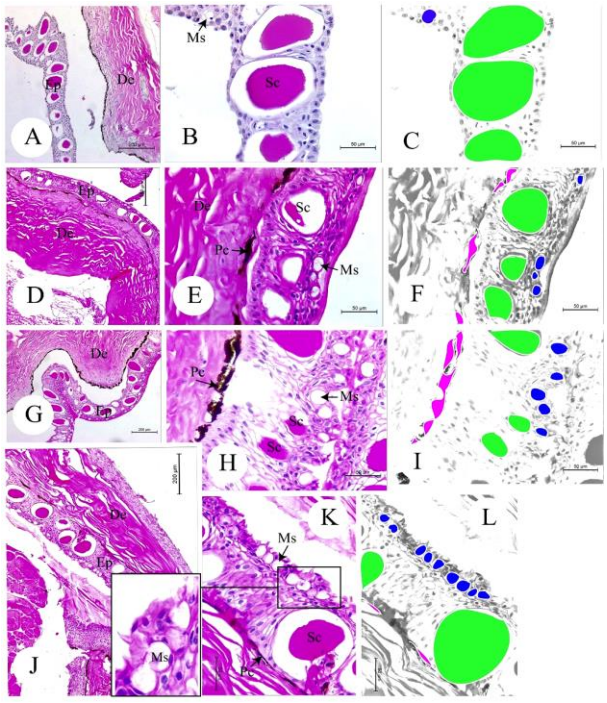

Figure 4. Representative light photomicrograph and schematic diagram showing the integument areas 4 (A-C), 5 (D-F), 6 (G-I) and 7 (J-L) of *Allenbatrachus grunniens*. Note: Ep = epidermis, De = dermis, Ms = mucus-secreting cells (blue color), Sc = sacciform cells (green color), Pc = pigment cells (pink color)

The high density of sacciform cells in the epidermis was visually observed in areas 3, 4, 5 and 6. The low density was displayed in area 7 (Figures 3D-3F and Table 1). We assume the anterior body is the center for protein secretion, an effective defense mechanism. The presence of this cell suggests that the sacciform cells are related to the production, storage, and release of the alarm substance, leading to an alarm reaction in phylogenetically close species (Smith, 1992) and a phagocytic function (Lufty, 1964).

The measured density of pigment cells showed variation by skin area (Table 1). We noted the high density in areas 2, 3, and 6, whereas the low density was in areas 4, 5, and 7. They should contribute to the varied coloration of the toadfish along the body as an adaptive function, which aids in various ways, including camouflage, aggressive purposes, and courting patterns (Kottler *et al.*, 2014).

Table 1. Summary of the densities of sacciform cells and pigment cells in *Allenbatrachus grunniens* integument

Integument area	Cell types	
	Sacciform cells	Pigment cells
1	2	2
2	2	3
3	3	3
4	3	1
5	3	1
6	3	3
7	1	1

Note: 0 cell = 0 score, 1-2 cell = 1 score, 3-4 cells = 2 score, 5-7 cells = 3 score, 8-10 cells = 4 score and 10+ cells = 5 score

3.3 Comparative number density reactions of the cells to different salinities

The density of mucus-secreting cells differed between the samples collected in December 2016 and January 2017 (Figure 5). The differences were significant in areas 3, 4, 5, and 7 ($P < 0.05$). The difference in salinity, rather than other seasonal factors, is likely responsible for the observed changes in the density of mucus-secreting cells because these samples were collected in two consecutive months. This is a novel finding that has not yet been widely confirmed. Sathorn *et al.* (2021) reported that the average density of mucus-secreting cells was dramatically increased according to the salinity level in female *Poecilia mexicana* gill, likely reflecting its osmoregulatory function. However, it is noted that the mucus-secreting cells serve as the site of protection surrounding the environment with biotic and abiotic factors and are believed to play an important role in innate immunity against pathogenic microbes (Dash *et al.*, 2008, 2018).

Hence, we speculate that these morphological features may be related to the potential role of mucus in ion regulation. This has been demonstrated by several approaches, including *in situ* measurements of ion gradients using ion-selective electrodes and measurements of the diffusion coefficients of key ions in the mucus (Shephard, 1982). Therefore, further experimental studies are required to gain a greater understanding of the roles of the activity of the mucus-secreting cells and other cells.

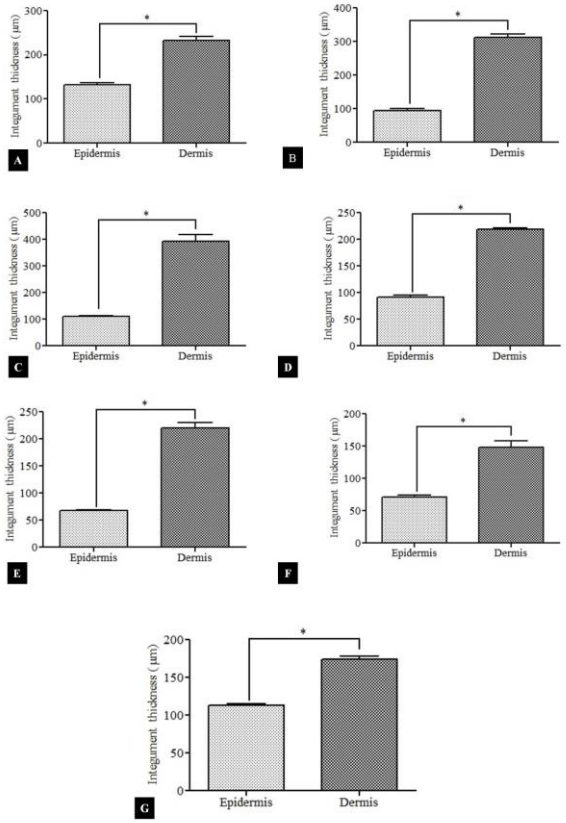

Figure 5. A comparison of skin thickness of *Allenbatrachus grunniens* integument. Values are given as mean ± SE; significant difference called at $*P<0.05$
Note: area 1 = upper jaw (A), area 2 = frontal part (B), area 3 = lateral part of the middle body (C), area 4 = lower jaw (D), area 5 = chin (E), area 6 = posterior part of the pectoral fin (F) and area 7 = caudal part (G)

4. Conclusions

Our observations revealed that the integument system of *A. grunniens* in the estuarine condition comprises two layers: epidermis and dermis, as seen in other teleosts. The density of mucus-secreting cells was changed depending on the salinity conditions, suggesting that this is an adaptation of the fish to live in estuarine environments. Therefore, further analyses of the function between mucus-secreting cells and different salinities in a laboratory study will be useful for understanding the adaptation of this toadfish to the estuarine environment.

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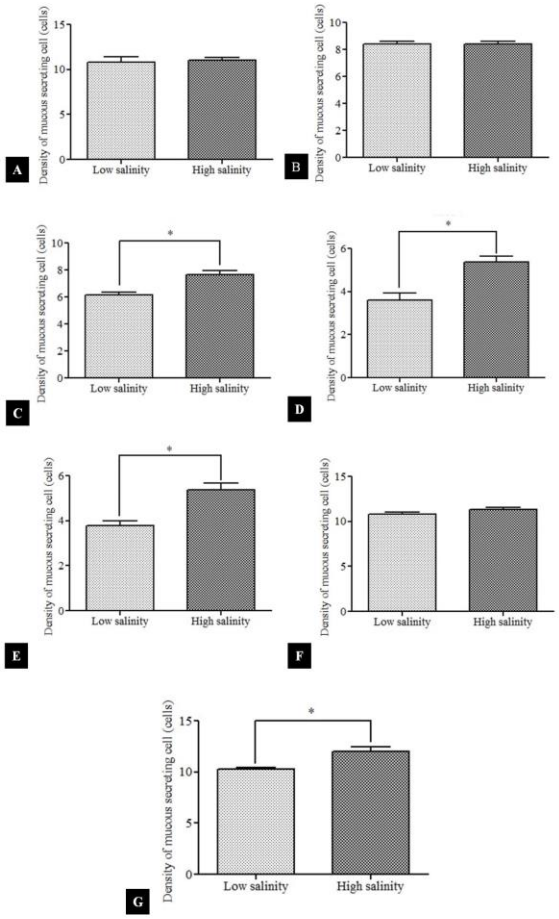

Figure 6. The density of mucus secreting cells of *Allenbatrachus grunniens* integument. Values are given as mean ± SE; significant difference called at **P*<0.05. :[a,b] indicate a statistically significant difference.
Note: area 1 = upper jaw (A), area 2 = frontal part (B), area 3 = lateral part of the middle body (C), area 4 = lower jaw (D), area 5 = chin (E), area 6 = posterior part of the pectoral fin (F) and area 7 = caudal part (G)

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