Characterization of the skin mucus in the common octopus *Octopus vulgaris* (Cuvier) reared paralarvae

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Abstract

The O*ctopus vulgaris* farming is impaired by the high mortality of the paralarvae during the first month of life. Several factors have been investigated in this regard, but no data exist on the body surface mucus, which represents the interface with the outside environment. This study included morphometric analysis and glycoconjugates characterization of skin mucus in reared *Octopus vulgaris* paralarvae during the first month of life. Four types of mucous cells were distinguished: mucous 1 (m1) and mucous 2 (m2) cells were scattered in the mantle epidermis, mucous 3 (m3) and mucous 4 (m4) in the epithelium surrounding the sucker. Except for the presence of fucosylated and neutral glycoconjugates in all mucous cells, each cell type expressed a characteristic glycopattern. m2 and m4 contained also suphate and acid non-sulphate glycans, m3 lacked sulphate glycoproteins. Lectin histochemistry showed that mantle mucous cells (m1, m2) expressed GlcNAc and lactosamine terminating glycans. m2 also contained GalNAc terminal or penultimate to sialic acid. m3 was distinguished by mannosylated glycans terminating with lactosamine and m4 by $\alpha 2,6$ sialoglycans. Glycoproteins terminating with lactosamine, Gal$\beta 1,3$GalNAc, and $\alpha 1,6$-linked fucose were a common feature of paralarvae surface layer. Morphometry revealed a significant decrease of m1 and m2 abundance during the first month of life, afterwards the reared paralarvae died. Since the glycopattern did not change during the investigated period, the mantle mucous cells abundance could be related to the *Octopus vulgaris* paralarvae survival.

Introduction

The cephalopod *Octopus vulgaris* is an important candidate for aquaculture although the farming is impaired due to the high mortality of the small planktonic hatchling,[1] conventionally called paralarvae.[2] Several authors have suggested that the transition from yolk utilization to active predation is a critical period in the early life history of cephalopods due to low rates of survival.[3-5] The high mortality problem has been related to the diet and its nutritional content. A nutritional imbalance in the proteins, lipids, minerals and other nutrients of the artificial feed has been pointed as one of the reasons for this high mortality.[6] Several factors have been investigated about the paralarvae survival such as swimming and feeding behaviour, ontogenetic changes in size and shape, arm growth, water circulation, den availability, food type.[1,6-9]

The skin of cephalopods is a complex system involved in a number of functions, such as lubrication, protection against mechanical damage, microbes, viruses, or proteolytic degradation, osmotic and ionic regulation, oxygen and nutrient uptake, sensitivity, camouflage and communication.[6,10,11] As in other aquatic organisms, several of these functions are mediated by glycoproteins constituting the mucus, a gel-like and viscous substance, secreted by mucous cells.[12-14] Among the species belonging to phylum Mollusca, the mucus has been extensively studied in gastropods and bivalves.[15-18] Concerning the cephalopods, mucus secreting cells have been observed in the skin of adults.[10,19-21] However, skin morphological changes have been described for chromatophores, photophores and Kölliker's organ during the growth of the cephalopods paralarvae and in particular in the genus *Octopus*.[5,10,22,23] Thus, despite the epidermis represents the outermost tissue directly interacting with environment and the first line of body defense, to date nothing is known about its mucus content along the *O. vulgaris* paralarva body, including the suckers. These structures play a key role in a variety of crucial processes for the paralarvae survival such as the grasping, manipulating and investigating objects and suction process.[6]

This study dealt with the morphological features and the glycoconjugate patterns of the skin from reared *O. vulgaris* paralarvae, in order to provide new insights about this critical period of the early life of this species.

Materials and Methods

Animals

Paralarvae of *Octopus vulgaris* from Maricoltura Mattinatese (Mattinata, FG, Italy) in partnership with Acquacoltura Jonica (San Vito, TA, Italy) were collected during aquaculture trials financially supported by F.E.P. 2007-2013 - Reg. CE n. 1198/2006 - "Reproduction and breeding of the common octopus, *Octopus vulgaris*". Mated *O. vulgaris* females (n=12) were kept in captivity using the technology described by Moxica *et al.*[24] and a mean of three hundred thousand paralarvae were obtained from spontaneous spawning. All the paralarvae were kept in captivity according to Iglesias *et al.*[1] Briefly, about two thousands of recently hatched (day 0) paralarvae were transferred to a 1 m³ PVC

circular tank (120 cm of diameter) provided with filtered seawater (1 μm) at a concentration of 2 individuals L⁻¹ with black wall and white bottom. Mean water temperature was 22.5°C (19.6-22.9°C), salinity 35 (34.2-35.7) and a 24 h light cycle was provided with two 36W daylight fluorescent tubes, resulting in an intensity of 600-1000 lux on surface. Levels of dissolved oxygen, nitrites and ammonium were measured daily. All tanks were provided of a close water circuit with central aeration during the first week and microalgae (*Chlorella sp., Isochrysis galbana* and *Chaetoceros sp.*) were added daily in order to feed the remaining preys in the culture tank, to keep them in the best nutritional condition. Then, the water system was partially open (10 L min⁻¹) 4 h per day, with a central outlet provided with a 300 μm filter. The tank bottom was cleaned by siphoning every 3 days. Paralarvae diet consisted of adult *Artemia* (1-4 mm TL and 0.05-0.1 individuals mL⁻¹) cultivated at 25°C during a week with a commercial Selco liquid HUFA (Inve Technologies, Dendermonde, Belgium) and enriched for 24 h with *Chlorella sp.*

Sampling and histology processing

Fifty paralarvae were collected from June to August 2013 at 1-4, 11-14 and 21-28 days after hatching and labelled as P01, P02 and P03, respectively. According to guidelines of European Directive 201/63/UE concerning the humane killing of animals in research and to Villanueva and Norman,[6] after collection the paralarvae were anesthetized in a 2% ethanol-seawater and fixed in 4% (v/v) PBS-buffered paraformaldehyde for 24 h at 4°C. Then the samples were dehydrated through a graded alcohol series and embedded in paraffin wax. Serial sections (4 μm thick) were cut and, after dewaxing with xylene and hydration in an ethanol series of descending concentrations, were stained with Hematoxylin-Eosin for morphological and morphometric studies and, by means of conventional histochemical procedures or lectin histochemistry for glicoconjugate characterization.

Conventional histochemistry

For the general staining of carbohydrates Periodic acid-Schiff (PAS) was performed.[25] Acidic glycans were detected with Alcian Blue pH 2.5 (AB pH 2.5).[26] Combined High iron diamine-Alcian Blue pH 2.5 (HID-AB pH 2.5) was used to stain simultaneously sulphated. (brown-black) and non-sulphated. (blue) acidic glycans.[27] Nuclei stained with Mayer's Hematoxylin. Details were about histochemical techniques can be found in Scillitani *et al.*[28] All the chemicals were from Sigma-Aldrich (St. Louis, MO, USA).

Lectin histochemistry

Binding of ten FITC-labelled lectins (Vector Laboratories, Burlingame, CA, USA) was tested to investigate the composition and distribution of oligosaccharidic chains in mucins (Table 1 and references therein[29-38]). Sections were incubated for 1 h at room temperature with the lectin solution in HEPES and subsequently rinsed in the same buffer and mounted in Fluoromount (Sigma-Aldrich). Each experiment was repeated twice on each sample.

Controls for lectin labelling were performed by i) substitution of lectin with HEPES alone; ii) incubation in lectin with inhibitory sugar added (0.2-0.5 M); iii) binding to the egg envelope of the toads *Bombina pachypus* and *Bufo bufo*, the mucins of which are demonstrated to be labelled by the tested lectins.[39,40]

Lectin-binding was observed in epifluorescence under 495 nm light emission. Photos were taken by a Nikon DMX 1200 camera coupled with a Nikon Eclipse 600 photomicroscope (Nikon Instruments SpA, Calenzano, FI, Italy).

Morphometry and statistical analysis

Four types of PAS positive muciparous cells (two in the mantle skin and two in the arms skin) were detected during the investigated periods (P01, P02, P03). The number of each muciparous cell type was counted on 30 microphotograph fields randomly detected in PAS-Hematoxylin stained sections using a 60x lens, and the cell abundance was expressed as mean numbers of mantle muciparous cells/100 μm and as number of mucous cells per sucker.

Image analysis was performed by ImageJ software.[41] Statistical evaluations were performed separately for each cell type. Descriptive statistics (arithmetic mean, standard deviation, sample variance, standard error, kurtosis, skewness) were computed for each sample. The normality of sample distributions was tested by Shapiro-Wilk tests and the homogeneity of sample variances were tested by Levene's tests. The significance of variation among mean values was evaluated by both parametric one-way Analysis of Variance (ANOVA) and non-parametric Kruskal-Wallis test (KW). Post-hoc pairwise comparisons allowed to detect the pairs of sample values which differed significantly and were performed with both parametric Tukey's Honestly-Significant-Difference (THSD, related to ANOVA) and non-parametric Dwass-Steel-Chritchlow-Fligner

Table 1. Lectin used, their sugar specificities and the inhibitory sugars used in control experiments.

Lectin abbreviation	Source of lectin	Binding specificity	μg/mL	Inhibitory sugar concentration
WGA	*Triticum vulgaris*[29]	(GlcNAcβ1,4)n	20	0.5 M GlcNac
GSA II	*Griffonia simplicifolia*[30]	GlcNAc	10	0.5 M GlcNac
SNA	*Sambucus nigra*[31]	Neu5Acα2,6Gal/GalNAc	20	0.2 M Neu5Ac
PNA	*Arachis hypogaea*[32]	Galβ1,3GalNAc	10	0.2 M Gal
RCA I	*Ricinus communis*[33]	Galβ1,4GlcNAc	20	0.2 M Gal
SBA	*Glycine max*[34]	GalNAc	20	0.2 M GalNAc
Con A	*Canavalia ensiformis*[35]	D-Man, D-Glc	20	0.1 M MαM
AAA	*Aleuria aurantia*[36]	Fucα1,6GlcNAc-βNAsn; Fucα1,3, Fucα1,4	10	0.2 M L-Fuc
UEAI	*Ulex europaeus*[37]	Fucα1,2	10	0.2 M L-Fuc
LTA	*Tetragonolobus purpureus*[38]	L-Fucα1,6GlcNAc; L-Fucα1,2Galβ1,4[L-Fuc1,3] GlcNAcβ1,6R	20	0.2 M L-Fuc

Asn, asparagine; Fuc, fucose; Gal, galactose; GalNAc, N-acetylgalactosamine; Glc, glucose; GlcNAc, N-acetylglucosamine; MαM, methyl-α-mannopyranoside; Man, mannose; Neu5Ac, N-acetylneuraminic (sialic) acid.

(DSCF, related to Kruskal-Wallis) tests. Significance for probability computed from tests was set at P<0.01. Statistical computations were generated by the Real Statistics Resource Pack software release 4.3.[42] All data were expressed as means ± standard error (SE).

Results

Morphology

The gross outer morphology of *O. vulgaris* paralarvae displayed relatively short arms, a large buccal mass, two large eyes and several chromatophores distributed in the skin covering the mantle and arms (Figure 1A).

The epidermis covering the mantle of paralarvae showed a simple columnar epithelium constituted by epitheliocytes and mucous cells (Figure 1B). The epidermis was crossed by several Kölliker's organs (Figure 1B). The head portion of paralarva body contained well developed and differentiated arms. The ventral side of each arm showed at least three suckers which were constituted by two "chambers": the infundibulum and the acetabulum (Figure 1C). The infundibulum was in the outer part and the acetabulum in the inner part of the sucker (Figure 1C). The infundibulum is the exposed portion of the sucker and the acetabulum is a spheroidal cavity that opens into the infundibulum through an orifice (Figure 1C). The epithelium covering the infundibulum was simple cuboidal, whereas the epithelium lining the acetabulum as well as the space interposed between adjacent suckers had a simple squamous appearance (Figure 1C).

Conventional histochemistry

The results of histochemistry methods are summarized in Table 2. The epitheliocytes of both the mantle and arms showed no cytoplasmic staining with all the used histochemical methods. As for the skin, api-cal surface it displayed PAS positivity in the mantle and suckers (Figure 2 A,B), whereas it showed AB 2.5 staining in the mantle (Figure 2C) and HID reactivity in the cuticle of the sucker infundibulum. Four different types of mucus-containing cells (named m1, m2, m3, m4) were distinguishable based on their appearance, localization and histochemical reactivity (Figure 2, Table 2). m1 and m2 cells showed columnar shape
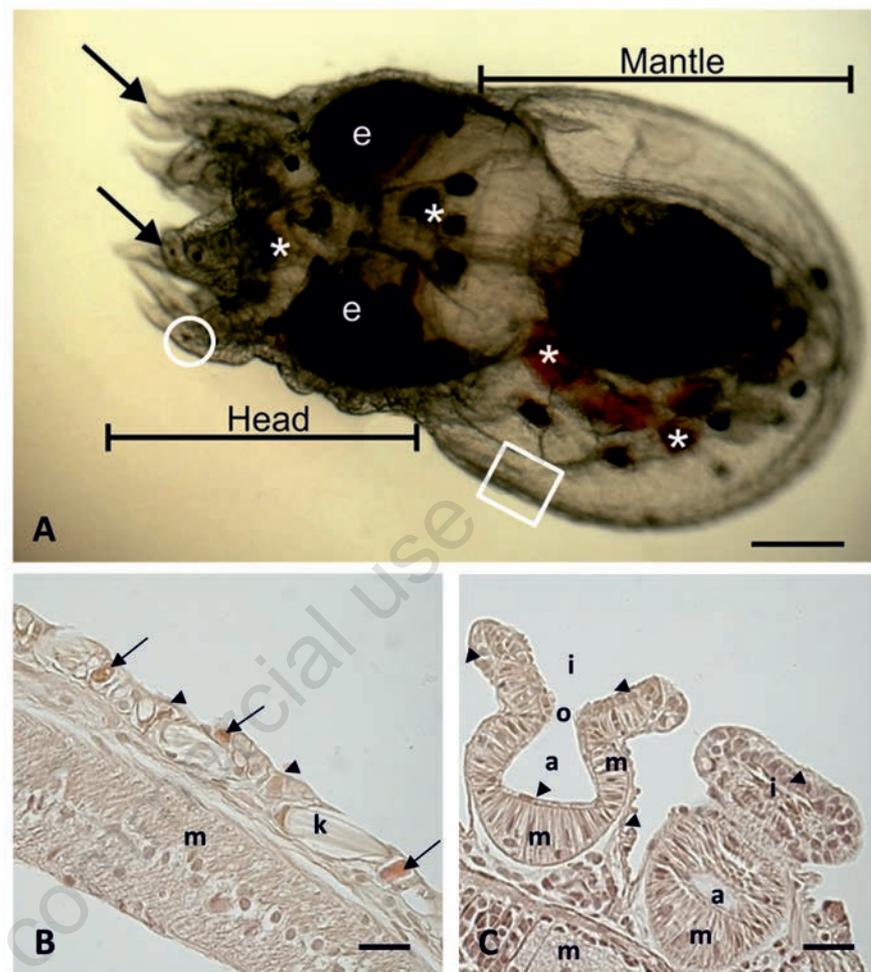

Figure 1. Gross morphology of a 2 days-old *Octopus vulgaris* paralarva (A) and (B,C) histological sections from squared and circled areas of A, respectively. A) Dorsal view of a paralarva showing short arms, two large eyes and several chromatophores distributed in the mantle and arms skin. B) Light micrograph of the mantle displaying columnar epitheliocytes and mucous cells scattered in the epidermis, Kölliker's organ organs and muscle wall. C) Light micrograph exhibiting the infundibulum and the acetabulum of two suckers. a, acetabulum; i, infundibulum; k, Kölliker's organ; m, muscle fibers; o, orifice; arrows, arms; arrowhead, epithelium; small arrow, mucous cells; e, eye; *chromatophores. B,C) H&E staining. Scale bars: A) 0.18 mm; B,C) 20 µm.

Table 2. Conventional and lectin histochemistry staining pattern of the mantle skin and suckers of the reared *Octopus vulgaris* paralarvae.

	PAS	AB 2.5	HID	WGA	GSA II	SNA	PNA	RCA I	SBA	Con A	AAA	UEA I	LTA
as	+	+m	+i	+i	-	-	+m,i	+m,i	-	+a	+m,i	+i,a	+i
m1	+	-	-	+	+	-	-	+	-	-	+	+	+
m2	+	+*	+*	+	+	+	-	+	+	-	+	+	+
m3	+	+	-	+	-	-	-	+	-	+	+	-	-
m4	+	+	+*	+	-	+	-	-	-	-	+	-	-

a, acetabulum; as, apical surface; i, infundibulum; m, mantle; m1, mucous type 1 cell; m2, mucous type 2 cell; m3, mucous type 3 cell; m4, mucous type 4 cell; *occasional positive cells; –, no staining; +, presence of reactivity.

and were in the mantle (Figure 2 A,C,E). m1 cells were coarse-grained PAS positive cells (Figure 2A), whereas m2 cells were fine-grained cells displaying strong PAS positivity (Figure 2A). Some m2 cells showed moderate affinity with both AB 2.5 (Figure 2C) and HID (inset of Figure 2E) staining. m3 and m4 cells were found in the epithelium surrounding the suckers. m3 cells were roundish in shape and were scattered in the epithelium surrounding the infundibulum (Figure 2 B,D,F) and strongly stained with PAS (Figure 2B) and AB 2.5 (Figure 2 D,F). m4 were round cells scattered in the epithelium surrounding the acetabulum zone of the sucker (Figure 2B, D,F). They displayed PAS (Figure 2B) and AB 2.5 (Figure 2D) positivity, although some of them exhibited HID positivity (Figure 2F). No staining variation was detected in the mantle and arm structures during the investigated periods. Kölliker's organs showed weak staining with the used methods (Figure 2 A,C,E).

Lectin histochemistry

The results of lectin histochemistry are summarized in Table 2 and illustrated in Figures 3 and 4.

N-acetylglucosamine residues (WGA, GSA II)

WGA bound to all the mucous cell types (Figure 3 A,B) as well as to the cuticle of the sucker infundibulum (Figure 3B). GSA II reacted with the m1 and m2 cells of the mantle (Figure 3C), whereas did not react with the mucosal cells and epithelium lining the arms (Figure 3D).

Sialic acid residues (SNA)

SNA, specific for NeuNAcα2,6 Gal/GalNAc terminal residues, linked the m2 and m4 cells but not the other mucous cells and the mantle and arms surface (Figure 3E, F).

Terminal galactose residues (PNA and RCA I)

PNA reacted with the apical surface of the epitheliocytes lining the mantle (Figure 3G) and with the cuticle of sucker infundibulum (Figure 3H). This lectin did not reveal binding sites in any mucous cells. RCA I bound m1 and m2 cells in the mantle (Figure 3I), m3 cells in the arms (Figure 3J) as well as the mantle surface and the cuticle lining the sucker infundibulum (Figure 3K).

N-acetylgalactosamine residues (SBA)

SBA displayed binding sites in m2 cells of the mantle (Figure 3L) and no reactivity in the arms (Figure 3M).

Terminal/internal mannose/glucose residues (Con A)

Con A did not bind the mantle whereas revealed high mannose glycans in the arms at level of the m3 cells and the cuticle of sucker acetabulum (Figure 4A).

Terminal fucose residues (AAA, UEA I, LTA)

AAA linked to all mucous cell types as well as to the surface of the mantle and sucker infundibulum (Figure 4 B,C). UEA I bound the m1 and m2 cells (Figure 4D) as well as the internal surface of the sucker (Figure 4E). LTA showed a similar binding pattern except for the lack of reactivity in the sucker acetabulum (Figure 4F).

The lectin histochemistry reactions did not change during the investigated periods and they were considered reliable because the negative-control procedures gave no reactivity (Figure 4G).
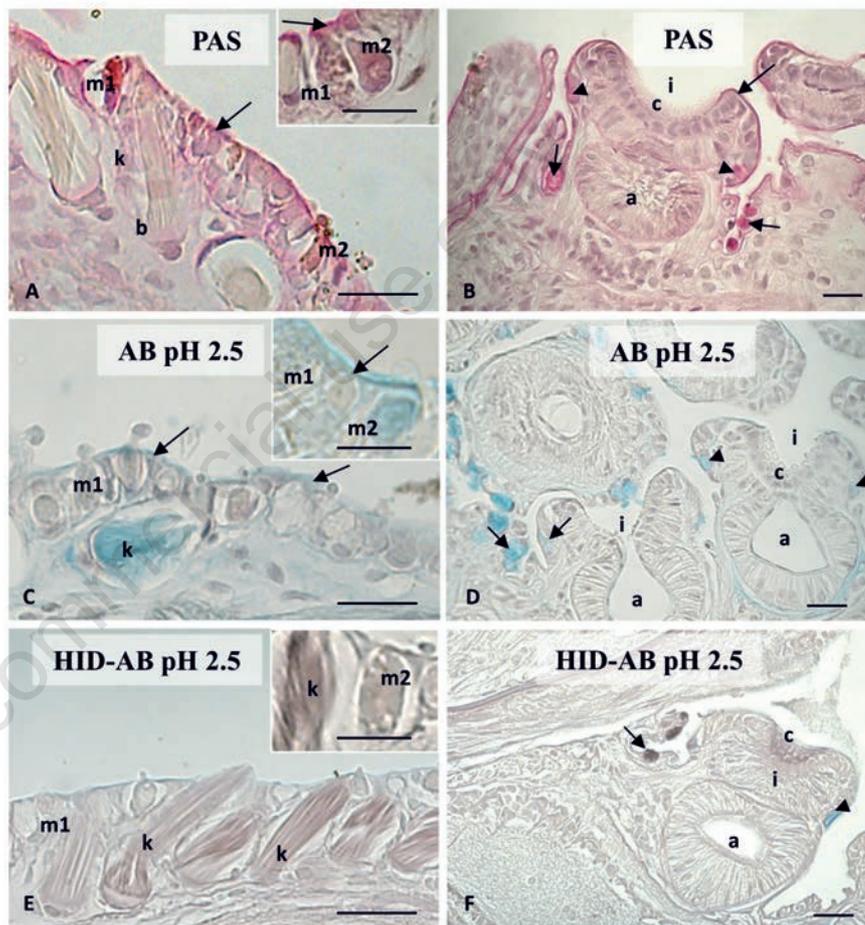

Figure 2. Skin of *Octopus vulgaris* paralarvae stained with PAS (A,B), Alcian Blue pH 2.5 (AB 2.5) (C,D) and HID-AB 2.5 (E,F) methods. A) PAS staining of a P01 paralarva mantle showing positivity of the m1 and m2 cells and the apical surface of epidermis (large arrow); inset: detail of coarse- and fine-grained granules in m1 and m2 cells, respectively. B) PAS positivity of m3 cells (arrowhead), m4 cells (small arrow), and the apical surface of arm epidermis (large arrow) and the cuticle (c) of a P02 paralarva sucker. C) AB2.5 staining of the mantle from a P02 paralarva showing positivity of the epidermis surface (large arrow) and Kölliker's organ fibers (k); m1 and m2 cells were usually unstained although m2 cells were occasionally stained (inset). D) AB 2.5 positivity of m3 (arrowhead) and m4 (small arrow) cells in P03 paralarva arms. E) HID-AB2.5 procedure displaying HID positivity (brown staining) of the Kölliker's organ organ fibers (k) and occasional m2 cells (inset) (P03 paralarva mantle). F) HID-AB 2.5 procedure showing rare HID (brown staining) m4 cells (small arrow) and AB 2.5 (blue staining) m3 cells (arrowhead) (P02 paralarva arm). A, acetabulum; c, cuticle; I, infundibulum; k, Kölliker's organ fibers; large arrow, epithelial apical surface; m1, coarse-grained mucous cell; m2, fine-grained mucous cell; arrowhead, m3 cell; P01, P02, P03, 1-4, 11-14 and 21-28 days after hatching samples; small arrow, m4 cell. Scale bars: A-F) 20 µm; insets in A,C,E) 10 µm.
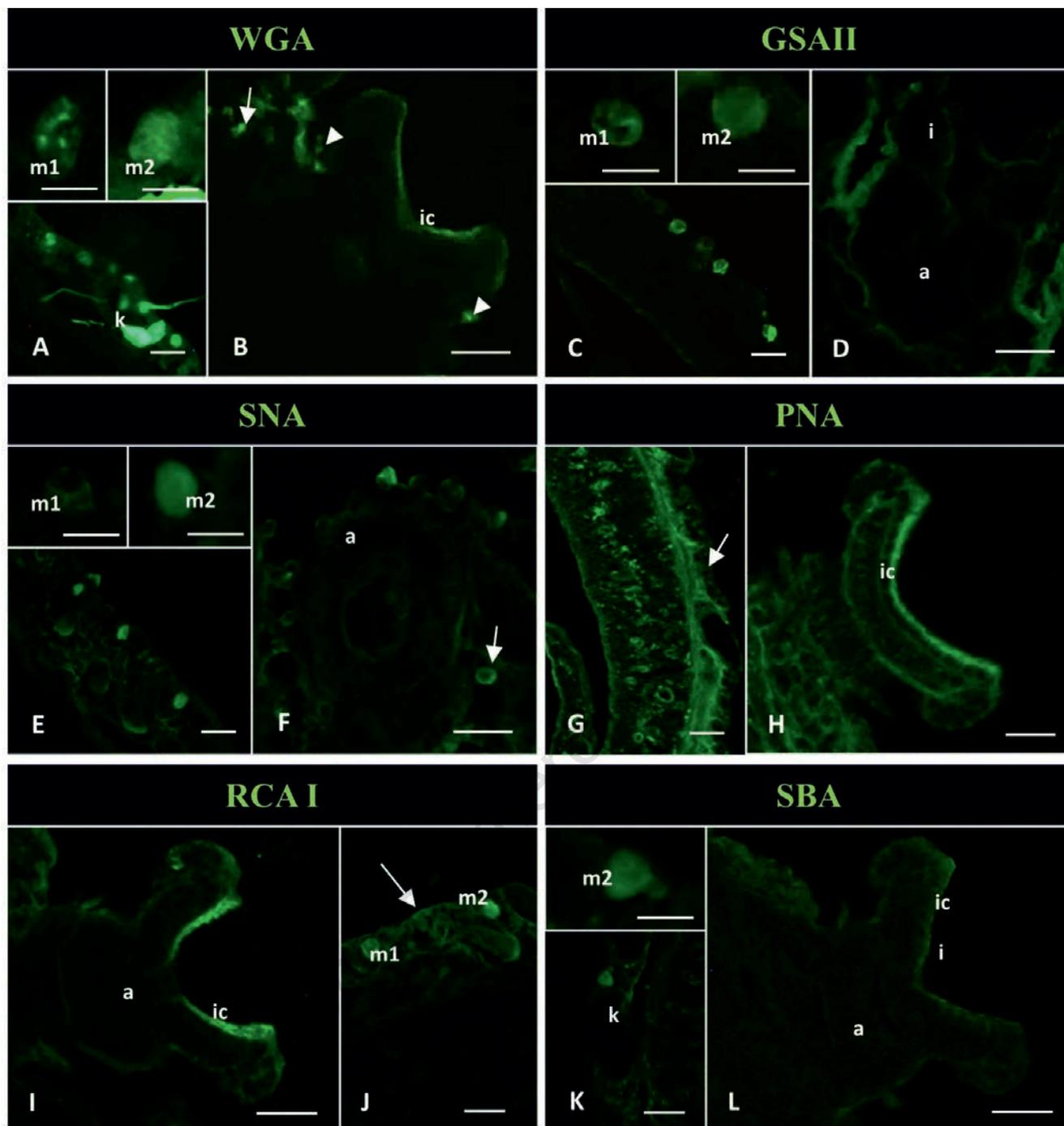
Figure 3. Lectin histochemistry of *Octopus vulgaris* paralarvae. A) WGA binding sites in m1, m2, Kölliker's organ of the mantle from a P01 paralarva. **B)** WGA reactivity in the infundibulum cuticle, m3 and m4 cells of a P01 paralarva sucker. **C)** GSA II staining of m1 and m2 cells from a P02 paralarva. **D)** GSA II negativity with sucker from same paralarva of C. **E)** SNA binding sites in m2 but not m1 cells from mantle of a P01 paralarva. **F)** SNA reactivity of m4 cells in same paralarva of E. **G,** PNA staining of mantle surface of a P03 paralarva. **H,** PNA affinity with the infundibulum cuticle of a P03 paralarva. **I)** RCA I staining of mucous cells and surface lining the mantle of a P01 paralarva. **J)** RCA I binding sites in m3 and infundibulum cuticle of a sucker from same paralarva of I. **K)** SBA binding with m1 cells of P01 paralarva mantle. **L)** SBA did not react with arms of the same paralarva of K. a, acetabulum; arrowhead, m3 cell; i, infundibulum; ic, infundibulum cuticle; k, Kölliker's organ; m1, coarse-grained mucous cell; m2, fine-grained mucous cell; P01, P02, P03, 1-4, 11-14 and 21-28 days after hatching samples; large arrow, mantle apical surface; small arrow, m4 cell. Scale bars: A-L) 20 µm; insets in A,C,E,K) 10 µm.

Statistical analysis

Descriptive statistical analysis was performed on the abundance of PAS-reactive m1, m2, m3 and m4 cell types and values obtained (expressed as M1, M2, M3 and M4, respectively) are showed in Tables 3 and 4. Figure 5 shows the histograms comparing mean values and standard errors for M1-M4 in the three samples. M1 and M2 are significantly different among samples. The M1 increases from P01 to P02 but decreases again in P03, whereas M2 decreases from P01 to P03. The relative abundance of the two cell types indicates that in P01 sample M2 outnumbers M1, but they attain similar values in P02 and P03. The deviations from normality tested by Shapiro-Wilk tests (*not shown*) were not significant except for M2 in P02

(W=0.7994, P=0.0006). Homogeneity of variances, assessed by Levene's tests (*not shown*), was revealed in M1 but not in M2 (P=0.0034). One-Way ANOVA and Shapiro-Wilk tests were performed on both M1 and M2 values among the samples and all the results are resumed in Table 5. Both tests revealed a high significant difference between M1 and M2 means among samples. The results of *post-hoc* comparisons of means between samples by THSD and DSCF tests are in Table 6. Most pairwise comparisons indicate significant differences, except for P01-P03 (THSD only) and P02-P03 (both THSD and DSCF).

The abundance of m3 e m4 sucker cells (indicated as M3 and M4, respectively) did not show deviations from normality tested by Shapiro-Wilk tests (*not shown*) and their variances had homogeneous values by

Levene's tests (*not shown*). The mean values did not vary significantly among samples (Figure 5 and Table 4). Thus, *post-hoc* comparisons were not performed.

Discussion

The body surface of aquatic organisms is covered with a protective mucous layer which represents the first interface between the animal and the environment. Therefore, the evaluation of the glycoconjugates composition of the skin mucus could give new data about the biology of octopus paralarvae which, due to high mortality rate, represent an impairment for the aquaculture. In this study, for the first time, we describe the glycan profile of the epidermis and suckers
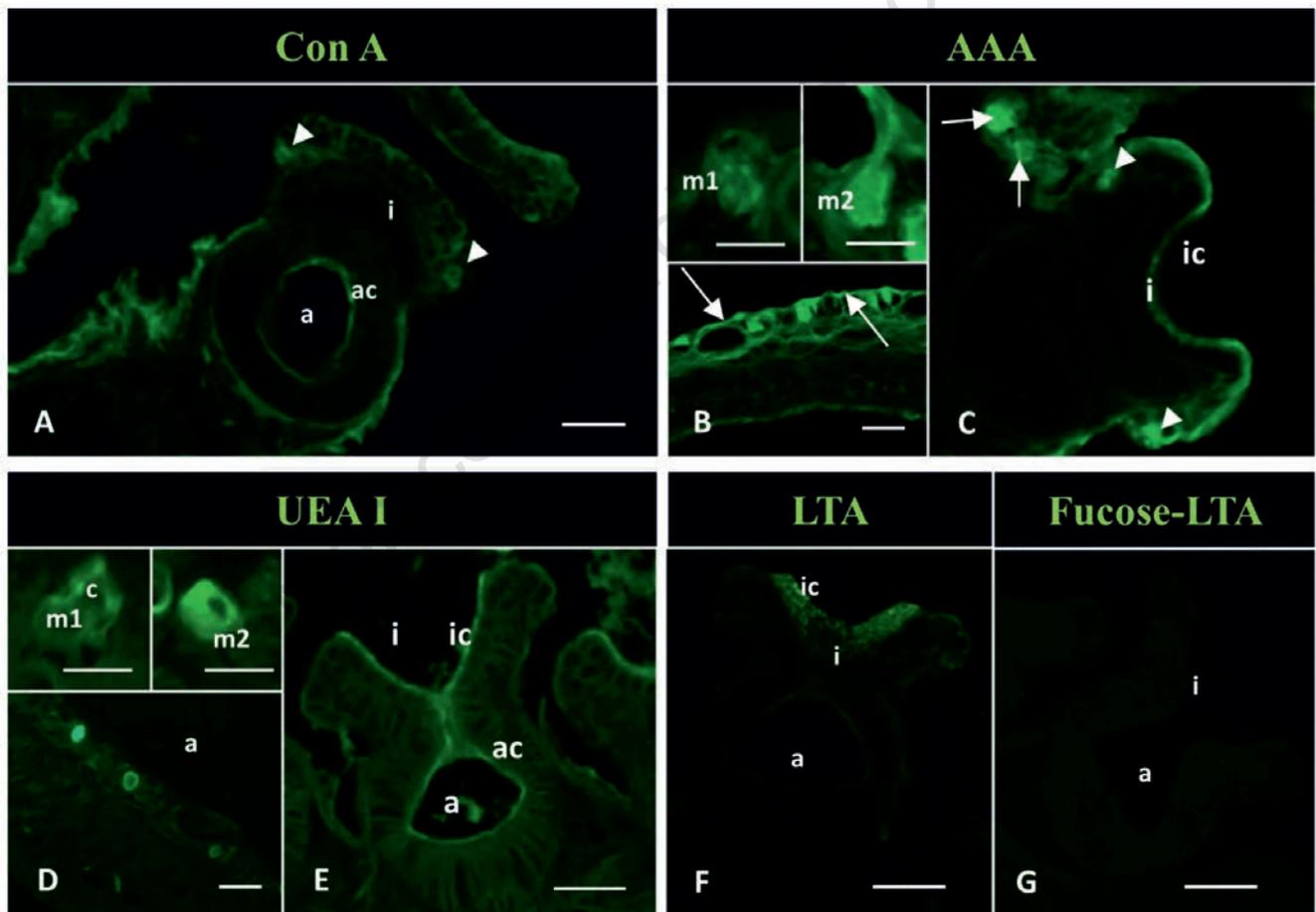
Figure 4. Lectin histochemistry of *Octopus vulgaris* paralarvae. A) Con A reactivity with m3 cells and acetabulum cuticle from a P01 paralarva. B) AAA staining of surface and m1 and m2 cells in P02 paralarva mantle. C) AAA binding with m3 and m4 cells and infundibulum cuticle of same paralarva of B. D) UEA I reactivity with the mucous cells of a P03 paralarva mantle. E) UEA I affinity with the infundibulum and the acetabulum cuticle of a P03 paralarva. F) LTA binding sites in the infundibulum cuticle of a P03 paralarva sucker. G) LTA un-reactivity with the P03 paralarva sucker when the inhibitory sugar was added (negative control). a, acetabulum; i, infundibulum; ac, acetabulum cuticle; ic, infundibulum cuticle; m1, coarse-grained mucous cell; m2, fine-grained mucous cell; arrowhead, m3 cell; large arrow, mantle apical surface; small arrow, m4 cell; P01, P02, P03, 1-4, 11-14 and 21-28 days after hatching samples. Scale bars: A-G) 20 μm; insets in B and D) 10 μm.

in the reared *O. vulgaris* paralarvae.

The cephalopod epidermal mucus-secreting cells have received little attention compared to other mollusks.[18] However, the diversity of mucous cells types found in the studied cephalopod species,[10,21] and the presence of a thin mucus-gel film surrounding the animal body[6] underlines its importance in the post-hatchling life.[43]

Morphological investigations revealed that the mantle was lined with the epidermis, which was a simple columnar epithelium constituted of epitheliocytes and two different types of mucous secreting cells: m1 and m2 cells. Both these secretory cells contained neutral glycoproteins (PAS staining) which presumably ended with GlcNAc (WGA and GSA II reactivity), lactosamine (RCA I affinity), and fucose (AAA, UEAI, and LTA binding). However, these two cell types produced different glycans since m2 cells synthesized also carboxyl (AB 2.5 positivity) and/or O-sulphate esters (HID staining) glycoproteins containing GalNAc and sialic acid (SBA and SNA binding, respectively).

This different glycosylation pattern could be related to differential functions of the mucins secreted by m1 and m2 cells. Interestingly, GalNAc is important for the O-glycosidic linkage of the saccharidic chains to the protein backbone (typical to secreted mucins[44]) and is often subterminal to sulphated and carboxylated residuals.[45,46] Concerning sialic acid, this sugar contributes to the net negative charge of glycans because it is typically found at the terminal position of glycans and it is involved in several important functions, such as intercellular interactions, transport of ions and molecules, viscosity of mucus, protec-

Table 3. Descriptive statistics for M1 and M2 numbers of mucous cells per 100 µm in the *Octopus vulgaris* paralarvae mantle skin.

Statistics	P01		P02		P03	
	M1	M2	M1	M2	M1	M2
Number of sections	46	46	21	21	21	21
Mean	1.002	2.860	1.422	1.359	0.969	0.836
Maximum	1.554	4.533	2.214	2.792	2.134	1.469
Minimum	0.484	1.062	0.899	0.731	0.268	0.429
Range	1.070	3.471	1.315	2.062	1.866	1.039
Standard deviation	0.253	0.834	0.324	0.508	0.510	0.242
Sample variance	0.064	0.691	0.105	0.258	0.260	0.058
Standard error	0.037	0.123	0.071	0.111	0.111	0.053
Kurtosis	-0.292	-0.512	0.727	2.910	-0.371	1.410
Skewness	0.175	0.239	0.848	1.764	0.573	0.971
Rank sum	1911	2931	1466	605	539	380

Table 4. Descriptive statistics for M3 and M4 numbers of sucker mucous cells in the paralarvae of *Octopus vulgaris*.

| Statistics	P01		P02		P03	
	M3	M4	M3	M4	M3	M4
Number of suckers	24	24	28	28	27	27
Mean	11.83	10.79	11.89	11.14	10.93	10.78
Maximum	15	15	15	15	15	15
Minimum	8	8	8	8	8	8
Range	7	7	7	7	7	7
Standard deviation	2.73	2.25	2.23	2.30	2.15	2.38
Sample variance	7.45	5.04	4.99	5.31	4.61	5.64
Standard error	0.56	0.46	0.42	0.44	0.41	0.46
Kurtosis	-1.60	-0.58	-1.17	-1.25	-0.86	-0.93
Skewness	-0.40	0.59	-0.20	-0.07	0.58	0.53
Rank sum	1016.00	933.50	1213.00	1187.50	931.00	1039.00
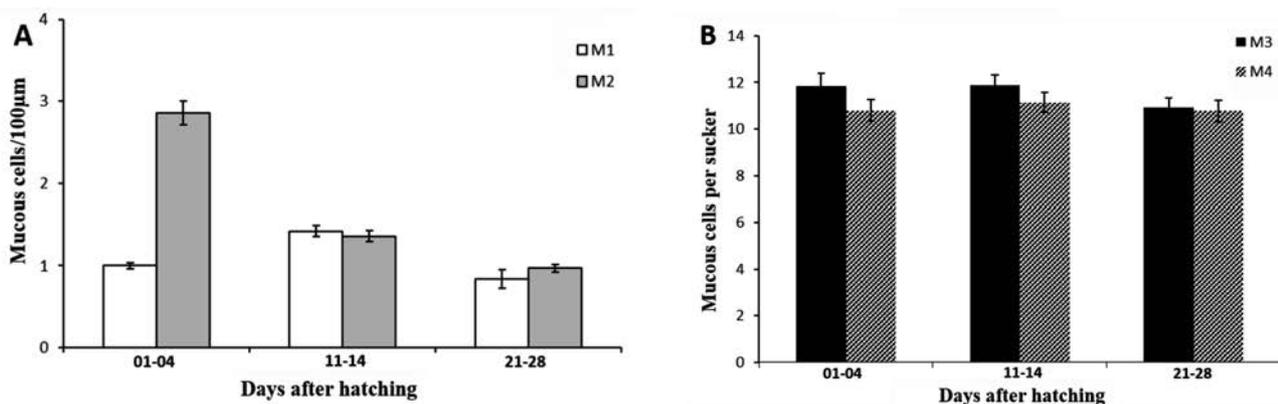
Figure 5. Histograms showing mantle and suckers mucous cells abundance in *Octopus vulgaris* paralarvae. Values are expressed as mean ± SE. M1, M2, M3, M4 indicate the abundance of m1, m2, m3 and m4 cells, respectively.

tion from microorganisms or interactions with symbionts,[47] and its alterations characterize several pathologies in vertebrates including human.[48] The presence of the sialic acid is an unusual finding in mollusks, where it is usually substituted by muramic acid.[45,49] Differently from the other monosaccharides, the backbone of this sugar can exhibit pronounced chemical diversity in structure and linkage so that all the known sialic acids constitute the sialome.[50] Thus, sialome analysis could be a useful tool for monitoring health conditions of paralarvae from hatching to settlement. Furthermore, the sulphated and fucosylated glycoproteins (HID, AAA, UEA I and LTA reactivity) could play an important role in microbial

interactions.[49] A morpho-functional interpretation of our results requires comparison with literature data, but this is difficult because of both the absence of such investigations and the different terminology used in reported studies.[10,51] Ultrastructural analysis of the *O. vulgaris* epidermis revealed the presence of three type of mantle secretory cells (type 1, 2, and 3) but no data concerning the type of secreted mucosubstances.[51] In sepiolids, the skin of *Euprymna scolopes* exhibited two types of secreting cells such as ovate cells and goblet cells producing sulphate and neutral glycoproteins, respectively.[19] Moreover, in the *Sepia esculenta* skin[21] three types of secreting cells (A, B, and C cells) were observed.

All these cells secreted neutral glycoconjugates and type A produced also acidic glycans.

The morphometric analysis showed that the trends of m1 and m2 cells number were significantly different, because m1 cells increased during the first two weeks and decreased during the fourth week after hatching, whereas m2 cells gradually reduced during the first month after hatching. However, interestingly the number of both mucous cell types decreased at the end of the month. This suggests two different hypotheses: i) the mucus secretion at level of the mantle changes at the end of the first month after hatching; ii) the reduction in the number of mucous cells could cause some

Table 5. Results of One-Way ANOVA and Kruskal-Wallis tests for comparisons of means between M1 and M2 numbers of mucous cells per 100 μm of the *Octopus vulgaris* paralarvae mantle skin.

M1	Test			SS	df	MS	
	One-Way ANOVA						
		Sources	Between groups	3.9659	2	1.9829	
			Within groups	6.1505	85	0.0724	
			Total	10.1163	87	0.1163	
		F					27.4042
		P					0.0000
	Kruskal-Wallis						
		H			2		32.6394
		P					0.0000
M2	**Test**			**SS**	**df**	**MS**	
	One-Way ANOVA						
		Sources	Between groups	64.7213	2	32.3607	
			Within groups	41.4948	85	0.4882	
			Total	106.2161	87	1.2209	
		F					66.2892
		P					0.0000
	Kruskal-Wallis						
		H			2		56.3832
		P					0.0000
M3	**Test**			**SS**	**df**	**MS**	
	One-Way ANOVA						
		Sources	Between groups	0.0002	2	0.0003	
			Within groups	0.0200	76	0.0003	
			Total	0.0202	78	0.0003	
		F					0.3923
		P					0.6773
	Kruskal-Wallis						
		H			2		0.7498
		P					0.6873
M4	**Test**			**SS**	**df**	**MS**	
	One-Way ANOVA						
		Sources	Between groups	0.0004			
			Within groups	0.0069			
			Total	0.0073			
		F					2.4013
		P					0.0974
	Kruskal-Wallis						
		H			2		3.9857
		P					0.1363

SS, sum of squares; df, degrees of freedom; MS, mean squares; F, value of Fisher's statistics; H, value of H statistics; P, value of probability associated to each test.

change in the glycan/molecular composition of mucous layer of the skin which can be related to the high mortality rates. However, the *O. vulgaris* paralarvae used in this study showed a growth pattern in line with the data reported in previously studies[8] (the body length ranged from 1.7±0.4 mm to 3.1±0.5 mm at four days and twenty-eight days from hatchling, respectively) and they exhibited the same morphological features described on wild animals[23] with no signs of anatomical damage.

The mantle surface exhibited neutral glycan and acidic non-sulphated glycans (PAS and AB 2.5 positivity) as well as glycoproteins terminating with Galβ1,3GalNAc (PNA), Galβ1,4GlcNAc (RCA I) and fucose linked α1,6 to GlcNAc or α1,3 to N-acetyllactosamine (AAA). This suggests that both m1 and m2 cells participate in the production of mucus layering the mantle surface. In sepioids the epidermal mucus layer has been related to the sticking of the sand for camouflage[19] as well as in the reduction of friction during swimming.[21] Since in *O. vulgaris* the relative importance of lubrication in swimming and adhesive interactions with the substrate probably change from a planktonic to a bottom-dwelling life style, we could hypothesize that the variation in abundance of m1 and m2 cells observed from early to older post-hatchlings could significantly affect these ecological requirements.

The arms exhibited suckers consisting of the infundibulum and acetabulum chambers which were connected through a constricted orifice. The epithelium lining the infundibulum consisted of cuboidal cells, whereas that covering the acetabulum was squamous. These features differ from those observed in the sucker of adult individuals. Kier and Smith[20] reported that the infundibulum is lined with a tall columnar epithelium and the acetabulum with a simple cuboidal one. This feature, along with the absence of the circumferential marginal

fold surrounding the infundibulum in suckers of adult individuals observed in previous studies,[6] suggests that the suction process in hatchling octopus paralarvae is not as effective as in adults.

Morphological studies on *O. vulgaris* paralarve suckers are scarce[23,52] and reports on the molecular composition of these structure are lacking. Kier and Smith[20] detected epithelial cells containing acidic mucopolysaccharides in a zone surrounding the sucker rim of adult *O. joubini*, but they did not describe typical mucus-secreting cells. In this study, we observed for the first time the presence of two mucous cells, defined as m3 and m4 cells, whose relative abundance did not significantly change during the investigated period. The m3 cells were scattered in the epithelium surrounding the infundibulum. This cell type contained neutral as well as acidic non-sulphated mucins constituted by mannosylated (N-linked) glycans (Con A affinity) containing GlcNAc (WGA reactivity) and terminating with lactosamine (RCA I staining) as well as with fucosylated residues binding AAA. m4 cells expressed neutral as well as acidic sulphated and non-sulphated mucins constituted of oligosaccharides containing GlcNAc (WGA affinity), sialic acid (SNA binding), and AAA-binding fucose.

The detected glycoproteins could be involved in the constitution of mucus surrounding the sucker rim to improve the watertight seal during the adhesion process. However, their differential composition suggests a different role of m3- and m4-secreted glycoproteins in this process. Moreover, the release of sulphated glycoproteins on the surface of the aquatic animal epidermis has been related to lubrication that is necessary in protecting the body against mechanical damages as well as in increasing of the viscosity to trap bacteria and other pathogens (reviewed by Rai *et al*.[53]). Interestingly, the morphometric analysis revealed that the number of m3 e

m4 cells is constant during the first month of life after hatching. This suggests that these cells, on the contrary of m1 and m2 cells, are not affected by the rearing conditions.

As for the sucker surface, the infundibulum surface was lined with the cuticle containing neutral and sulphated mucins which were constituted of GlcNAc, Galβ1,3GalNAc, lactosamine, and all investigated fucosylated glycans. Interestingly, the cuticle covering the acetabulum expressed a different glycan pattern compared to the infundibulum, since it lacked acidic glycans and expressed highly mannosylated glycans and α1,2-linked fucose. This finding agrees with the report that the sucker is covered by a chitinous cuticle which is particularly well developed on the infundibulum.[20] On this basis, we suppose that the differential glycan composition of infundibulum and acetabulum cuticle may differently aid the suckers in attaining suction adhesion. Different functions of the two compartments of the sucker have been reported in previous studies by different technical approaches.[54,55] The adhesive properties of mucus have been observed in other mollusks. Glue mucus in gastropodes is more rich in sulphated and acetylated sugars than trail mucus.[18,56,57]

In conclusion, the present study provides for the first time an insight into the glycoconjugates expressed in the secretory cells and the surface lining the mantle and the suckers of reared *O. vulgaris* paralarvae. In addition, the results provide information on the changes in the number of mucous cells scattered along the mantle of skin of *O. vulgaris* paralarvae during the first month of life. The presented results add information which could be useful in sustain pure as well aquaculture experimental researches aimed to better understand the physiology of this species during this critical stage of the life cycle.

Table 6. Results of Tukey's Honestly-Significant-Difference (THSD) and Dwass-Steel-Chritchlow-Fligner (DSCF) *post-hoc* tests for comparisons of means between M1 and M2 numbers of mucous cells per 100 µm of the *Octopus vulgaris* paralarvae mantle skin.

	S1	S2	THSD		DSCF	
			Value	P	Value	P
M1	P01	P02	-0.420	0.000	-9.768	0.000
	P01	P03	0.166	0.055	-20.118	0.000
	P02	P03	0.586	0.000	-6.848	0.000
M2	P01	P02	1.501	0.000	-24.257	0.000
	P01	P03	1.891	0.000	-25.136	0.000
	P02	P03	0.390	0.173	-3.149	0.067

S1-3, sample; P, value of probability associated to each test.

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