Histochemistry of goblet cells and micro-computed tomography to study the digestive system in the long-snouted seahorse *Hippocampus guttulatus*

Claudia Ofelio[a,*] claudiaofelio@gmail.com, Stefania Cohen[b], Dominique Adriaens[c], Giuseppe Radaelli[d], Alcira Ofelia Díaz[b]

[a]Departamento de Ecología y Recursos Marinos, Instituto de Investigaciones Marinas, Consejo Superior de Investigaciones Científicas (IIM-CSIC), Eduardo Cabello 6, 36208 Vigo, Spain

[b]Departamento de Biología. Instituto de Investigaciones Marinas y Costeras (IIMyC), FCEyN, CONICET- Universidad Nacional de Mar del Plata. Funes 3250, 7600 Mar del Plata, Buenos Aires, Argentina

[c]Ghent University, Evolutionary Morphology of Vertebrates, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium

[d]Universitá di Padova, Dipartimento di Biomedicina Comparata e Alimentazione, Viale dell'Universitá 16, 35020 Legnaro, Italy

[*]Corresponding author at: Claudia Ofelio, Instituto de Investigaciones Marinas, Consejo Superior de Investigaciones Científicas (IIM-CSIC), Eduardo Cabello 6, 36208 Vigo, Spain.

Abstract

Sygnathids are agastric teleosts (no stomach), relying on a digestive tract using different mechanisms to process and absorb nutrients. This results in a low digestion efficiency at

early stages, forming a mayor bottleneck in the rearing of these fish. In agastric species, the numerous goblet cells present in the oesophagus could be considered as a morphological adaptation that replaces a functional stomach, although the specialization and number of these cells is species-specific and vary throughout the development and intestinal region. The present study aim to characterize the biochemical composition of goblet cells and investigate the morphology of the digestive system during the ontogeny of *Hippocampus guttulatus* seahorses (from 0 to 60 days post-partum, dpp) in order to understand the mechanisms of nutrient digestion in a species that lacks gastric glands. Goblet cells (GC) appear in the digestive tract of *Hippocampus guttulatus* from birth on, secreting a large amount of acid (carboxylated) and neutral glycoconjugates (GCs) released in the buccopharyngeal cavity and oesophagus, while sulphated and neutral GCs are detected throughout the complete digestive tract of the seahorses. The midgut mainly secretes neutral and acid (carboxylated) GCs at the early stages (<15 dpp) and acid (sulphated) GCs at later stages, while the hindgut is characterized by the presence of carboxylated and highly sulphated GCs throughout development.

Based on the development of the digestive tract observed by 3D reconstructed µCT data, an increase in the intestinal absorption surface can be assumed. This increase is in correspondence with a change in the type of acid GCs observed in the midgut from 15 dpp. The present study demonstrates that the digestive system of *H. guttulatus* is functional at first feeding, although not fully developed. The different GCs secreted by the goblet cells in the digestive tract of *H. guttulatus* revealed that the glycosylation patterns vary according to the digestive region and stage of development. Neutral GCs could be involved in the digestion of simple substances (e.g. short chain fatty acids) in early stages of development, while in more advanced stages the change to acid GCs

would promote the absorption of proteins. A change in the secretion of goblet cells from 15 dpp, in coincidence with the observation of a progress increase in the intestinal absorption surface, would explain the better digestive efficiency observed from that age.

KEYWORDS: goblet cells; histochemistry, intestine; ontogeny; seahorses.

1. Introduction

The morphology and functionality of the digestive system in fish is species-specific, and it is related to feeding habits, taxonomy, body size, ontogeny and phylogeny (Banan Khojasteh, 2012; Wilson and Castro, 2010). Even if newborns develop directly or indirectly to become juveniles, the digestive tract undergoes several changes until becomes completely functional (Falk-Petersen, 2005).

According to its morpho-functional characteristic, the digestive tract of fish can be subdivided into four regions: headgut (buccopharynx), foregut (oesophagus), midgut (anterior intestine / stomach) and hindgut (distal intestine) (Wilson and Castro, 2010). These regions are always exposed to bacteria, parasites, toxins and viruses, which come from the luminal content (Neuhaus et al., 2007). As a defence system, a permanent mucous layer covers the intestinal epithelium in aquatic vertebrates. The mucous layer is composed of a highly hydrated gel that consists approximately of 95% of water and 5% of mucins, plus minor components like electrolytes (Díaz et al., 2008). Mucins are high molecular weight glycoproteins synthesized mostly by goblet cells (GC) of the digestive tract. Mucin glycoproteins can be classified into: a) gel-forming secretory mucins that are a fundamental component of mucus, responsible for their physical-chemical properties and b) transmembrane mucins that are expressed in the apical

membrane of all the epithelial cells that line the mucous membranes and represent the major component of the glycocalyx (Tano de la Hoz et al., 2017). In addition to the glycoproteins, the glycocalyx is constituted by glycolipids (Johansson et al., 2011). Both glycoproteins and glycolipids constitute the group of glycoconjugates (GCs). The specialization and number of goblet cells vary among the development and intestinal region (Domeneghini, 1998). Conversely, diet does not affect its biochemical composition (Fiertak and Kilarski, 2002; Ortiz-Delgado et al., 2012), even though the number of goblet cells could be related to the fish diet (Gargiulo et al., 1998). Depending on their biochemical composition, each type of GCs may exert a specific functional role such as nutrient absorption, lubrication, protection against proteolytic degradation, antimicrobial function, ionic and osmotic regulation (Bansil and Turner, 2006; Díaz et al., 2008; Domeneghini et al., 2005). In fish, goblet cells with neutral GCs (GCs with oxidizable vicinal diols) participate in the protection of mucosal folding against proteolytic degradation (Díaz et al., 2003) and their presence could also be related to absorption processes (Micale et al., 2008). The role of sialic acid has been related to protection, since it acts as a receptor of bacteria, binding strongly to them and thus preventing their adherence to the surface of the epithelium. The presence of sialic acid hampers viral and bacterial infections, preventing the viruses from recognizing their cellular receptors and hindering the attack of bacterial sialidase. The GCs with sulphated esters in the teleost mucus function mainly as lubricants, while the presence of sulphate groups has also been related to the increase in the acidity of the secretions, preventing bacterial colonization (Cohen et al., 2016).

The histochemistry of the digestive system has been widely studied in several teleost species (Cohen et al., 2014; Domeneghini et al., 2005; Kozarić et al., 2007; Pedini et al.,

2001; Sarasquete et al., 1995), but not so for seahorses. Seahorses (genus *Hippocampus*) are marine teleost belonging to the family Sygnathidae, which offspring undergo a direct development in the brooding structure (pouch) of their father. Upon release from the brood pouch, they change from an endogenous to an exogenous feeding regime, being able to feed on live prey from the first hours after release (Roos et al., 2011). In such agastric species, which lack of a stomach (Wilson and Orr, 2011), digestion mainly takes place in the intestine (Rønnestad et al., 2013). The digestive tract of seahorses appears at early stages as a short and straight tube, promoting the rapid evacuation of the ingested food (Ofelio et al., 2018; Randazzo et al., 2018). Research on the biology and rearing techniques of seahorses has considerably increased in recent years (Cohen et al., 2016) with the aim to reduce the pressure wild populations and provide sustainable hatchery procedures to satisfy the increasing demand for the international aquarium trade (Koldewey and Martin-Smith, 2010). Newborn seahorses are considered juveniles, with a completely developed and physiologically functional anatomy at the onset of exogenous feeding. So far, the high mortality of seahorses at early developmental stages has been related to nutritional factors and the digestibility of the diet (Blanco et al., 2015; Palma et al., 2014; Palma and Andrade, 2012). Nonetheless, a low digestive efficiency and absorption of nutrients from the diet may be due to an incomplete functionality of the digestive tract at those stages.

The European long snouted seahorse *Hippocampus guttulatus* is catalogued as data deficient in the IUCN Red List of Threatened Species (IUCN, 2016). Its breeding represents an alternative to the capture of individuals in the wild (e.g. for the supply to aquariums) and opens the possibility of reinforcement or restocking plans. In spite of recent studies carried out on *H. guttulatus* (Blanco et al., 2015; Palma et al., 2014;

Planas et al., 2012), the digestive functions and food absorption mechanisms are almost unknown, although they are an important factor to consider in order to improving conservational and breeding programs.

The aim of the present work is to i) characterize the biochemical composition and distribution of goblet cells in the four regions of the digestive tract of *H. guttulatus* during ontogeny and ii) investigate the morphology of the digestive system during the early development, in order to understand the mechanisms of nutrient digestion in a species that lacks gastric glands.

2. Material and Methods

2.1 Ethics

Sampling methods, animal maintenance and manipulation practices carried out in the present study were conducted in compliance with all bioethics standards of the Regional Government Xunta de Galicia (REGA ES 36057020 2001/16/FUN.BIOL.AN/MPO02) under the Spanish Governmental Regulation RD 1201/2005, 10th October and approved by CSIC Bioethics Committee.

2.2 Rearing system and feeding regime

The brood stock consisted of an F1 generation of adult *H. guttulatus* maintained in 630L aquaria units, assembled as four autonomous modular tanks (A, B, C and D) in a semi-opened seawater system. Each unit consisted of three sub-units of 160 L each (85 x 75 x 50 cm) arranged in a T-shape (Planas et al., 2008).

Photoperiod and temperature regimes during the breeding season were established at 16L:8D and 18.5ºC, respectively, simulating the natural conditions from June to

September in Galicia (NW Spain). The water flow rate was established at 6 L min^{-1} and two pipes provided aeration of each aquarium unit. Salinity and pH levels were maintained at 37 and 8.0 ± 0.2 respectively. A 20 W fluorescent tube (4.000 K) provided a light intensity of about 850-1050 lx at each aquarium unit. Plastic plants and ropes (about 12 mm in ø) anchored to small stones were placed on the bottom of the aquaria. A 10% of total water volume was renewed daily before first feeding and uneaten food and faeces removed.

Newborn seahorses (5.5 ± 1.0 mg in wet weight and 15.1 ± 1.1 mm in length) from five batches were distributed across thirteen 30 L pseudo-Kreisel aquaria with gentle aeration at initial densities of 5 individuals L^{-1} (Blanco et al., 2014). The aquaria were connected to a semi-open recirculation system, including a degasifying column and two 50 L bio-filters containing mechanical (up to 20 µm) and biological filters, aerators and skimmers. The seawater was pumped from the bio-filters to 36 W UV light units and then to 50 L reservoir tanks.

Water temperature was maintained at 19 ± 1°C using an inlet heating system (HC300/500 A; HAILEA, Guang- Dong, China). A 16L:8D photoperiod regime was applied and the light source consisted of 20 W Power Glo T5 fluorescent lamps (Hagen Group, Valencia, España). Gentle aeration was supplied as described by Blanco (2014). *Artemia* cysts (*EG* Grade *INVE* Aquaculture, Belgium) were incubated daily at 28°C for 24 h and hatched nauplii transferred to 5 L buckets (100 mL^{-1}). Two daily doses of *Artemia* nauplii (1 mL^{-1} per dose) and one dose of cultivated copepods (0.7 mL^{-1}) were delivered to newborn seahorses from 0 to 10 days post-partum (dpp). From 11 to 30 dpp, juveniles were fed with two daily doses of 24 h enriched *Artemia* metanuplii (1 mL^{-1}). The enrichment was carried out on a daily mixture of 10^7 cells mL^{-1} of both

Phaeodactium tricornutum and *Isochrysis galbana* microalgae, spry-dried Spirulina (0.02 g L^{-1}) and Red Pepper (0.1 g L^{-1}, BernAqua, Belgium). A mixture of 24, 48 and 72 h enriched metanauplii were supplied from 31 dpp until the end of the study (60 dpp). Before each feeding, uneaten food and faeces were siphoned and 30% of aquarium volume daily renewed.

Copepods, *Acartia sp.*, were cultivated in 500 L tanks (1-3 mL^{-1}) on a mixture of *Isochrysis galbana* and *Rhodomonas len*s microalgae (10^6 cells mL^{-1}).

Broodstock were daily fed with adult (>96 h) enriched *Artemia sp.* (*EG* Grade *INVE* Aquaculture, Belgium) maintained in 100 L units at 26ºC during 15-25 days, and wild mysidaceans (*Leptomisys* spp. and *Sirella* spp.) three times day^{-1}.

2.3 Seahorse sampling

A total number of 174 individuals proceeding from five batches of F2 generation were sampled at 0, 5, 10, 15, 20, 30 and 60 dpp, each time before the first feeding dose (n 12-61 at each sampling time).

Sampled seahorses were individually anesthetized with an overdose of MS-222 0.1 g L^{-1} (Sigma-Aldrich, Germany), washed with tap water and photographed. Subsequently, the seahorses were gently dried to remove the excess of water, and weighted on a MC210P Sartorius microbalance (± 0.01 mg). Afterwards, sampled specimens were individually fixed in 4% buffered formaldehyde.

2.4 Histochemistry

Fixed samples were individually transferred to histology cassettes, washed over-night under running tap water and rinsed with distilled water. Cassettes were immersed in

10% formic acid (Sigma-Aldrich, Germany) during 5 days for bone decalcification. Tissues were dehydrated in a graded series of ethanol and then paraffin wax embedded. A rotary microtome was used to cut 5 µm thick thin sections.

Conventional histochemistry techniques were applied for the biochemical characterization of goblet cells in histological sections (table 1). Tissues were stained with Periodic Acid Shiff (PAS) to demonstrate periodate reactive vicinal diols and glycogen. Before PAS treatment, sections were subjected to an enzymatic digestion with α-amylase for glycogen identification control. Furthermore, the combined technique Alcian Blue pH 2.5 followed by Periodic Acid Shiff (AB pH 2.5/PAS) allowed the identification of acid (AB positive), neutral (PAS positive) and mixed (AB/PAS positive) secretion in one section, evidencing acid GCs with carboxyl group and O-sulphate esters (turquoise), periodate reactive vicinal diols (magenta), and GCs presence with carboxyl groups and O-sulphate esters together with periodate reactive vicinal diols (purple). Alcian Blue pH 2.8 was used to evidence GCs with carboxylic group and O-sulphates esters. Sulphated GCs were identified with AB pH 1.0 solution, and highly sulphated GCs with AB pH 0.5, whereas GCs with carboxyl group and O-sulphated esters were evidenced with Toluidine Blue pH 5.6 and sulphated GCs at TB pH 4.2. Astra blue was used to evidence sialylated GCs; Periodic Acid–Borohydride reduction – Saponification – Periodic Acid–Schiff reaction (PA*/Bh/KOH/PAS) was applied to demonstrate sialic acid residues with substituents at C7, C8 or C9 and O-acyl sugars.

Image analysis was performed using CorelDraw® graphic Suite X8, ImageJ-Fiji and Adobe Photoshop CS6.

2.5 µ-CT scan and 3D reconstruction

A number of 26 individual were sampled at dpp 0, 5, 10, 15, 20, 30 (n = 3) and 60 (n = 2), fixed in 4% buffered formaldehyde and transferred to 1% PBS (GE Healthcare Life Sciences, USA). To perform the microcomputed tomography (µCT-scan) in order to visualise the digestive system, specimens were removed from PBS and submerged in 2.5 % solution of phosphomolybdic acid (PMA) during a period ranging from one day to one week. PMA has proven to allow detailed visualisation of soft tissues, including digestive tract anatomy using µCT scanning (Descamps et al., 2014). Samples were scanned at the Centre for X-ray Tomography at Ghent University (UGCT) with the HECTOR µCT-scanner, built and developed in collaboration with the company X-Ray Engineering (www.xre.be). HECTOR was used with the following setup: 80 kV tube voltage and 1501 projections over 360°. The pixel pitch of the detector was 210 µm and the resulting voxel sizes for the seven age groups were respectively 4.00, 6.00, 7.00, 15.00 16.20, 12.45 and 22.00 µm. The radiographic images were acquired using a PerkinElmer (BioTech company, London) camera. All µCT-data were processed to generate graphical 3D-reconstructions of the digestive tract by using AMIRA 5.5.0 software (Visage Imaging, San Diego, CA, USA). For the 3D processing, a *Volume rendering* module was applied to explore data and clear the image from the structure supporting the specimens during the scan. The lumen and intestinal epithelium of the digestive tract were selected manually with the *Segmented Editor* from every ten radiographic image projections. A different colour was assigned at each structure (lumen and intestinal epithelium) of the digestive tract. After that, the selections were extrapolated to the rest of the images and a smooth 3D reconstruction was generated by

using the *Surface Gen* module. Images of the 3D reconstructions were obtained by *Snapshot* tool.

3. Results

At hatching, the digestive tract appears as a straight, tubular structure, differentiated into four regions: the buccopharyngeal cavity (headgut), the oesophagus (foregut), the midgut (proximal intestine) and the hindgut (distal intestine). The histochemical analyses reveal a different GCs composition depending on the intestinal region and the developmental stage (Table 2-5). With the α-amylase technique, the presence of periodate reactive vicinal diols and glycogen is demonstrated when tissues are positive for the PAS reaction.

3.1 Buccopharyngeal cavity (headgut)

The histochemical reactivity in the buccopharyngeal cavity is mainly due to goblet cells types rather than to glycocalyx reactivity. Goblet cells strongly react to PAS at each sampling time, suggesting the presence of GCs with oxidizable vicinal diols and glycogen from birth (Fig. 1a).

From 5 dpp, the Astra Blue reaction shows a moderate amount of sialylate GCs, and it is possible to observe scarce amounts of GCs substituted at C7, C8 and/or C9, according to the weak reaction to PA*/Bh/KOH/PAS technique. The AB pH 2.5/PAS allows the identification of three types of goblet cells throughout the ontogeny: GCs with oxidizable vicinal diols (PAS-positive), cells producing carboxylated and sulphated GCs (AB-positive) and cells with mixed secretions (AB/PAS positive). The

metachromatic reaction with TB at both 5.6 and 4.2 pH demonstrates large amounts of acid GCs in the mucous cells of the headgut during the development.

3.2 Oesophagus (foregut)

From release, mixed and acid GCs are detected in a moderate amount in the glycocalyx and in goblet cells (Fig 1b). The foregut is strongly reactive to PAS (Fig. 1c, h); defining the presence of GCs with oxidizable vicinal diols and glycogen both in goblet cells and in the glycocalyx on the apical surface of epithelial cells. Contrary to the headgut, a large amount of substituted sialic acids in sialylated GCs is observed by the strong reaction to Astra Blue and PA*/Bh/KOH/PAS (Fig. 1d, g), while the negative reaction to AB at a low pH suggests the absence of sulphated GCs. From 10 dpp, the goblet cells show a strong reactivity with AB pH 2.8, and a moderate reactivity with AB pH 1.0 (Fig. 1e) and 0.5, indicating the presence of carboxylated and sulphated GCs. As in the headgut, the TB staining at both 5.6 (Fig. 1f) and 4.2 pH evidences the presence of metachromatic goblet cells in this region throughout development. At 20 dpp, granular apical goblet cells are strongly PAS positive (Fig. 1h), revealing at 30 and 60 dpp a large variety of goblet cells secreting neutral, acid and mixed GCs (Fig. 1j).

3.3 Midgut

At release, the oesophagus directly joins to the anterior intestine through an abrupt change from simple to columnar epithelium. At this stage, the midgut is still undifferentiated, appearing as a straight and short tube (Fig. 2 a, b, c), lacking a proper stomach. It represents the longest portion of the digestive tract. The apical surface of gut epithelial cells presents an acidophilic zone that matched with the brush border. A

strong reaction to the AB/PAS staining is observed in the glycocalyx of the cell membrane that forms the brush border of the midgut, indicating the presence of neutral GCs (Fig. 3a), while the reaction to Astra Blue shows sialylated GCs in goblet cells (Fig. 3b). At 15 dpp, the midgut increases in size, acquiring an S-shape and starts to coil to form the first loop (Fig. 2d), which was clearly observed at 20 dpp (Fig. 2e). The presence of GCs with O-group substituted in C7, C8 and/or C9 is confirmed by the strong reaction to PA*/Bh/KOH/PAS. The presence of neutral GCs is confirmed by a strong reaction to PAS in goblet cells (Fig. 3c, e). Similarly, sulphated and sialylated GCs are intensively marked with AB at different pH (Fig. 3d) and with Astra blue stains, respectively. To best accommodate the intestinal coil into the visceral cavity, a second loop is formed at 30 dpp (Fig. 2f). At 60 dpp, the midgut has substantially increased in size and is packed into a double eight shape (Fig. 2g). In addition, it is possible to observe mixed and neutral GCs with the combined AB pH 2.8/PAS staining (Fig. 3f).

3.4 Hindgut

The hindgut is compartmentalised from the midgut through an ileocecal valve and characterized, from release, by numerous goblet cells (Fig. 4a). Sulphated and carboxylated GCs are present in large amounts in the goblet cells of the distal intestine, as confirmed by the strong reactivity to AB at different pH (Fig. 4b). These acid GCs are also observed with the TB staining at both 5.6 and 4.6 pH. During the development, the glycocalyx is strongly reactive to PAS, while the goblet cells are PAS positive only from 10 dpp (Fig. 4c, d). Moreover, the glycocalyx is also weakly reactive to PA*/Bh/KOH/PAS, while the goblet cells remain unstained with this technique. The

AB 2.8/PAS technique reveals that the glycocalyx is characterized by neutral and mixed GCs, while the goblet cells are constituted only by acid GCs (Fig. 4e). In both structures, a moderate amount of sialylated GCs is identified by the positive reaction with Astra Blue (Fig. 4f).

4. Discussion

The present study characterises the histochemical composition and morphological structure of the digestive system in *H. guttulatus* during early ontogeny, from 0 till 60 days post-partum (dpp). From the early stages on, the digestive tract comprises a headgut, foregut, midgut and hindgut. Seahorses being agastric fish, lack a stomach and gastric digestion throughout their development (Wilson and Castro, 2010). Goblet cells were present all over the digestive tract of *H. guttulatus* and the production of different mucous content has been demonstrated through ten histochemical techniques.

In teleosts, neutral glycoconjugates (GCs) have been found mainly in the stomach and intestine, being involved in pregastric digestion (preventing from proteolytic damages and promoting the absorption of easily digestible substances) and functionality of the gastric glands (Díaz et al., 2003; Falk-Petersen, 2005; Gisbert et al., 2004). According to results obtained from agastric fishes (Falk-Petersen 2005; Micale et al. 2008; Ortiz-Delgado et al. 2012), digestive goblet cells are first seen in the buccopharyngeal cavity and oesophagus, interspersed in between epithelial layers. These goblet cells, secreting a high diversity of mucosubstances from birth on, with predominance of neutral and acid GCs, could be considered as a morphological adaptation to replace a functional stomach (Jaroszewska et al., 2008). A glycocalyx layer with neutral and acid GCs also covered

the apical surface of the intestinal epithelium of *H. guttulatus*. These GCs play an important role in the absorption process and in the transport of macromolecules through membranes (Zambonino et al., 2008). Neutral GCs have been linked to enzymatic digestion and the capacity to absorb easy digestible substances, such as short chain fatty acids and disaccharides (Gisbert et al., 2004; Micale et al., 2008). The acid GCs revealed in the present study were mainly carboxylated and sulphated in the buccopharyngeal cavity and sialylated in the oesophagus. In fish, acid GCs produced in the buccopharyngeal cavity and oesophagus play an important lubricant role increasing the mucus viscosity (due to the presence of sulphated GCs), and facilitating the entrapment of food particles (Gisbert et al. 2004; Micale et al. 2008). The GCs with sulphated esters in the teleost mucus would also function as lubricants. The amount of sulphate groups has been related to the increase in the acidity of the secretions, preventing bacterial colonization (Cohen et al., 2014). The primary role of sialic acid has also been related to bacteria protection, through a strong binding with their receptors and thus preventing the adherence to the surface of the epithelium (Messner et al., 2013). The mucosubstances produced by these cells in the midgut can help the displacement of food particles and protect the intestinal mucosa from mechanical injury (Kozarić et al., 2007), besides the length of the intestine and the mucosal folding might influence its absorptive capability (Zambonino et al., 2008).

Based on the increased intestinal length observed by 3D reconstructed µCT data, an increase in absorption surface can be assumed. This increase is in correspondence with a change in the type of GCs observed in the midgut from 15 dpp. Both transformations occur synchronously with an ontogenetic shift in prey preference from smaller (copepods) to larger preys (24h *Artemia* metanauplii) (Blanco and Planas, 2015). This

ontogenetic dietary shift could be associated with the change in goblet cells type, linked to a rapid increase in the size of ingested prey (Yúfera et al., 2014).

The histochemical complexity of goblet cells observed in the posterior intestine can be related to the multiple roles this part of the digestive tract play. The mucosubstances observed in the distal intestine of several teleost species increased in number during the ontogeny, being mainly involved in the lubrication for the ejection of faeces (Cohen et al., 2014; Pedini et al., 2001). Nevertheless, a high number of acid mucins was observed in the hindgut of *H. guttulatus* since birth, probably due to the early feeding activity of this species. In agreement with some authors (Domeneghini, 1998; Zambonino et al., 2008), sulphated GCs are secreted in the distal intestine, at first sight, as a result to ingestion and digestion of protein via pinocytosis, being involved in the regulation of protein and peptide transfer; as well as in the protection from bacteria and other pathogens.

4.1 Conclusions

It can be concluded that the digestive system of *H. guttulatus* is functional at first feeding, although not fully developed. The different GCs secreted by the goblet cells in the digestive tract of *H. guttulatus* revealed that the glycosylation patterns vary according to the digestive region and stage of development. Neutral GCs could be involved in the digestion of simple substances (e.g. short chain fatty acids) in early stages of development, while in more advanced stages the change to acid GCs would promote the absorption of proteins. A progressive increase of the intestinal absorption surface observed from 15 days could promote digestive and nutrient assimilation

capacities, enhancing the digestive efficiency of *H. guttulatus* compared to less developed stages.

Acknowledgments

We express our gratitude to Alex Chamorro from the IIM-CSIC of Vigo and Giovanni Caporale from the University of Padova for their technical support in the experimental rearing and histochemical processes. We are grateful to Barbara De Kegel and Ivan Josipovic from Ghent University for their technical support with the μ-CT scan.

Funding: This study was partially financed by the European Union (Erasmus+ fellowship) and by a grant awarded by the Spanish Ministry of Education, Culture and Sport.

Declarations of interest: none.

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Figure 1. Sagittal histochemical section of buccopharyngeal cavity and foregut in *Hippocampus guttulatus* from the day of partum (dop) until 60 days post-partum (dpp). **a**) PAS technique, buccopharyngeal cavity, positive cells in red (dop); **b**) AB/PAS technique in foregut, goblet cells secreting acid, neutral and mixed glycoconjugates, (5 dpp); **c**) PAS technique, foregut, positive cells in red. Detail of α-amilase technique in foregut, (5 dpp); **d**) Astra, technique in foregut, sialylated glycoconjugates in turquoise; (5 dpp); **e**) AB pH 1.0 technique in foregut, goblet cells with acid sulphated glycoconjugates in blue (10 dpp); **f**) AT pH 5.6 technique, metachromatic and orthochromatic goblet cells in foregut (20 dpp); **g**) PA*/Bh/KOH/PAS technique, goblet cells with sialic acids in foregut (20 dpp); **h**) PAS technique, asterisks mark granular goblet cells in foregut (20 dpp); **i**) AB/PAS technique, goblet cells with acid, neutral and mixed glycoconjugates (30 dpp); **j**) AB/PAS technique, goblet cells with acid, neutral and mixed glycoconjugates (60 dpp). *a*, acid glycoconjugates; *gl*, glycocalyx; *GC*, goblet cell; *m*, mixed glycoconjugates; *mt*, metachromasia; *n*, neutral glycoconjugates; *o*, orthochromasia.

Figure 2. Tridimensional reconstruction of the alimentary tract in *Hippocampus guttulatus* from the day of partum (dop) until 60 days post-partum (dpp). *FG*, foregut; *ICV*, ileocecal valve; *HD*, headgut; *HG* hindgut; *MG*, midgut. Lumen in violet/blue, intestinal epithelium in red-pink, asterisks indicate the position of intestinal loop during the ontogeny.

Figure 3. Sagittal histochemical section of midgut in *Hippocampus guttulatus* from the day of partum (dop) until 60 days post-partum (dpp). **a**) AB/PAS technique, glycocalyx of intestinal epithelium indicated by headarrow (dop); **b**) Astra technique, goblet cells with acid secretions in turquoise (dop); **c**) PAS technique, goblet cells with oxidizable vicinal diols and glycogen in red (20 dpp); **d**) Alcian Blue pH 0.5 technique, goblet cells with high sulphated esters in turquoise (20 dpp); **e**) PA*/Bh/KOH/PAS technique, goblet cells with sialic acids in red (20 dpp); f) AB/PAS technique, goblet cells with acid and mixed glycoconjugates (60 dpp). *gl*, glycocalix,; *GC*, goblet cells.

Figure 4. Sagittal histochemical section of hindgut in *Hippocampus guttulatus* from the day of partum (dop) until 60 days post-partum (dpp). **a)** Astra technique, goblet cells with sialylated glycoconjugates in turquoise (dop); **b**) Alcian Blue pH 0.5 technique, goblet cells with high sulphated esters in turquoise (5dpp); **c)** PAS technique, goblet cells with oxidizable vicinal diols and glycogen in red (10 dpp); **d**) PAS technique, goblet cells with oxidizable vicinal diols and glycogen in red (60 dpp); **e**) AB/PAS technique, goblet cells with acid glycoconjugates in turquoise (5 dpp); **f**) Astra technique, goblet cells with sialylated glycoconjugates in turquoise (dop). gl, glycocalix; *ICV*, ileocecal valve; *GC*, goblet cells.

Table 1. Histochemical staining used for the identification of glycoconjugates in *Hippocampus guttulatus* digestive tract.

Staining[a]	Summarized Protocol	Interpretation of staining reactions	References
PAS	Oxidation with periodic acid followed by Schiff's reagent.	GCs with oxidizable vicinal diols and glycogen: *Magenta*	McManus, 1948
α-amilase/PAS	Ptyalin incubation at 36°C during 45 min, previous to PAS reaction.	GCs with periodate vicinal diols: *Magenta*; Glycogen: *Transparent*	Fernandes et al., 2006
AB pH 2.5/PAS	Alcian blue pH 2.8 during 25 min followed by oxidation with periodic acid and Schiff's reagent.	Acids GCs: *Turquoise*; Neutral GCs: *Magenta;* Mixed GCs: *Purple*	Mowry, 1963
AB pH 2.8	Alcian blue pH 2.8 during 25 min.	Acid GCs with carboxyl group and O-sulphate esters: *Turquoise*	Lev and Spicer, 1964
AB pH 1.0	Alcian blue pH 1.0 during 25 min.	Acid GCs with O-sulphated esters: *Turquoise*	Lev and Spicer, 1964
AB pH 0.5	Alcian blue pH 0.5 during 25 min.	Acid highly sulphated esters: *Turquoise*	Lev and Spicer, 1964
TB pH 5.6	Toluidine blue in acetate buffer pH 5.6 during 2 min.	Acid GCs with O-sulphated esters and carboxyl group: *Orthochromasia Metachromasia*	Lison, 1953
TB pH 4.2	Toluidine blue in acetate buffer pH 4.2 during 2 min.	Acid GCs with O-sulphated esters: *Orthochromasia Metachromasia*	Lison, 1953
Astra Blue	Astra blue PH 2.5 during 30 min, sodium tetraborate during 10 min.	Acid GCs: *Blue turquoise*	Elie and Lecluse, 1986
PA*/Bh/KOH/PAS	Two-h oxidation at room temperature with 1% periodic acid, followed by reduction with sodium borohydride (PA-Bh). Then, saponification (KOH) and finally PAS reaction.	Sialic acid residues with O-acyl substitution at 7C, 8C or 9C and O-acyl sugars: *Magenta*	Reid et al., 1973

[a]AB, Alcian blue; Bh, borohydride; KOH, potassium hydroxide; PA, periodic acid oxidation; PA*, selective periodic acid oxidation; PAS, periodic acid Schiff reagent; PA*S, periodic acid/Schiff at low temperature and low pH (oxidation with 0.4 mM periodic acid in 1.0 M hydrochloric acid at 4°C); TB, Toluidine blue.

Table 2. Glycoconjugates composition in buccopharynx of *Hippocampus guttulatus* during the ontogeny (from 0 to 60 days post-partum, dpp).

	Headgut - Buccopharynx													
Staining	**Glycocalix**							**Goblet cells**						
dpp	0	5	10	15	20	30	60	0	5	10	15	20	30	60
PAS	-	-	1	-	-	-	-	-	3	3	2	3	3	2
PA/Bh/KOH/PAS	-	-	-	-	-	-	-	-	1	1	1	1	1	1
AB pH 2.8	2	-	-	-	-	-	-	2	2	1	1	2	2	2
AB pH 1.0	-	-	-	-	-	-	-	-	1	-	-	2	-	-
AB pH 0.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AB pH 2.5/ PAS	T3	-	-	-	-	-	-	T 2	T 2	P2	T 1	T2	T 2	T 2
								P2	P2	-	P2	-	P3	P 2
								M2	M2	-	M2	-	M2	M2
Astra Blue	-	-	-	-	-	-	-	2	2	3	2	2	2	2
TB pH 5.6	-	-	-	-	-	-	-	-	m 1	m 1	m 1	m 1	-	m 1
TB pH 4.2	-	-	-	-	-	-	-	-	m 2	-	-	-	-	-

M, magenta; P, purple; T, turquoise; m, metachromasia. Staining intensity: 1 slightly positive; 2 moderate; 3 strong.

Table 3. Glycoconjugates composition in foregut of *Hippocampus guttulatus* during the ontogeny (from 0 to 60 days post-partum, dpp).

	Foregut - Oesophagus													
Procedures	**Glycocalix**							**Goblet cells**						
dpp	0	5	10	15	20	30	60	0	5	10	15	20	30	60
PAS	-	2	2	2	2	2	-	-	2	2	2	2	2	2
PA*/Bh/KOH/PAS	-	-	-	-	-	-	-	3	3	3	3	-	-	3
AB pH 2.8	2	-	1	-	1	1	1	2	2	3	3	3	3	2
AB pH 1.0	-	-	-	-	-	-	-	1	1	2	1	1	1	-
AB pH 0.5	-	-	-	-	-	-	-	-	-	2	-	2	2	-
AB pH 2.5/ PAS	T 3	-	P 1	-	P 2	P2	P 2	T 2	T 2	T 2	T 2	T 2	T 2	T 2
								P 2	P 2	P 1	P 2	P 2	P 2	P 2
											M			
								M 2	M 2	-	1	M 1	M 1	M 1
Astra Blue	3	1	2	2	-	2	-	2	2	2	2	-	2	3
TB pH 5.6	-	-	-	-	o1	-	-	-	m 2	m 2	m 2	m 2	-	m 2
TB pH 4.2	-	-	-	-	o1	-	-	-	-	m 2	m 2	m 2	-	m 2

M, magenta; P, purple; T, turquoise; m, metachromasia; o, orthochromasia. Staining intensity: 1 slightly positive; 2 moderate; 3 strong.

Table 4. Glycoconjugates composition in midgut of *Hippocampus guttulatus* during the ontogeny (from 0 to 60 days post-partum, dpp).

	Midgut – Anterior Intestine													
Procedures	**Glycocalix**							**Goblet cells**						
dpp	0	5	10	15	20	30	60	0	5	10	15	20	30	60
PAS	1	2	2	2	2	2	2	2	2	2	2	2	2	2
PA/Bh/KOH/PAS	-	1	1	1	1	-	1	-	3	3	3	3	3	3
AB pH 2,8	1	2	2	1	1	1	1	1	2	1	3	3	3	3
AB pH 1.0	-	1	2	-	-	-	-	-	2	2	2	-	-	-
AB pH 0.5	-	2	-	-	-	-	-	-	2	-	2	2	2	-
AB pH 2.5/ PAS	P 3	P 2	P 2	P 2	P 2	P2	P 2	P 3	P 3	P 2	P 3	P 2	P2	P 2
	M 2	T 3	-	-	M 2	T 2	-	-	M3	-	M1		M1	M 1
								-	-	-	T2	T 2	T3	T 3
Astra Blue	2	2	2	3	2	2	2	3	2	2	-	2	2	2
	-	-	o 2	-	-	-	-	-	m 1	-	m 1	m 1	-	o 1
TB pH 5.6									o 1		o 2			
TB pH 4.2	-	-	-	-	-	-	-	-	-	m 2	m 1	m 2	-	-

M, magenta; P, purple; T, turquoise; m, metachromasia; o, orthochromasia.. Staining intensity: 1 slightly positive; 2 moderate; 3 strong.

Table 5. Glycoconjugates composition in the hindgut of *Hippocampus guttulatus* during the ontogeny (from 0 to 60 days post-partum, dpp).

	Hindgut - Posterior Intestine													
Procedure	**Glycocalix**							**Goblet cells**						
dpp	0	5	10	15	20	30	60	0	5	10	15	20	30	60
PAS	3	3	2	2	2	2	-	-	-	2	1	2	2	2
PA/Bh/KOH/PAS	-	1	1	1	1	-	1	-	-	-	-	-	-	-
AB pH 2.8	1	1	1	1	1	1	1	1	1	3	3	3	3	3
AB pH 1.0	-	-	-	-	-	-	-	-	-	2	2	2	2	2
AB pH 0.5	-	2	1	-	-	-	-	-	-	2	2	2	2	-
AB pH 2.5/ PAS	P 3	P 2	P 2	T2	P 2	P2	T2	T 3	T 2	T 2	T 2	T 2	T 2	T 2
	-	-	M2	P 2	M2		P2	-	-	-	-	-	-	P 2
Astra Blue	2	2	2	3	2	3	2	3	3	2	2	2	2	2
TB pH 5.6	-	o 1	-	-	-	-	-	-	m 2	m 2	m 2	m 2	-	m 2
TB pH 4.2	-	-	-	-	-	-	-	-	m 2	m 1	-	-	-	-

M, magenta; P, purple; T, turquoise; m, metachromasia; o, orthochromasia. Staining intensity: 1 slightly positive; 2 moderate; 3 strong.

Highlights

- The present study combine a traditional and innovative technique to evaluate the mechanisms of digestion of a species that lack of stomach.
- The histochemical complexity and glycosylation pattern in the digestive system vary in accordance with the developmental stage and digestive regions.
- Neutral glycoconjugates could be involved in the digestion of simple substances (e.g. short chain fatty acids) in early stages of development, while in more advanced stages the change to acid glycoconjugates would promote the absorption of proteins.
- A progressive increase of the intestinal absorption surface observed from 15 days could promote digestive and nutrient assimilation capacities, enhancing the digestive efficiency of *H. guttulatus* compared to less developed stages.

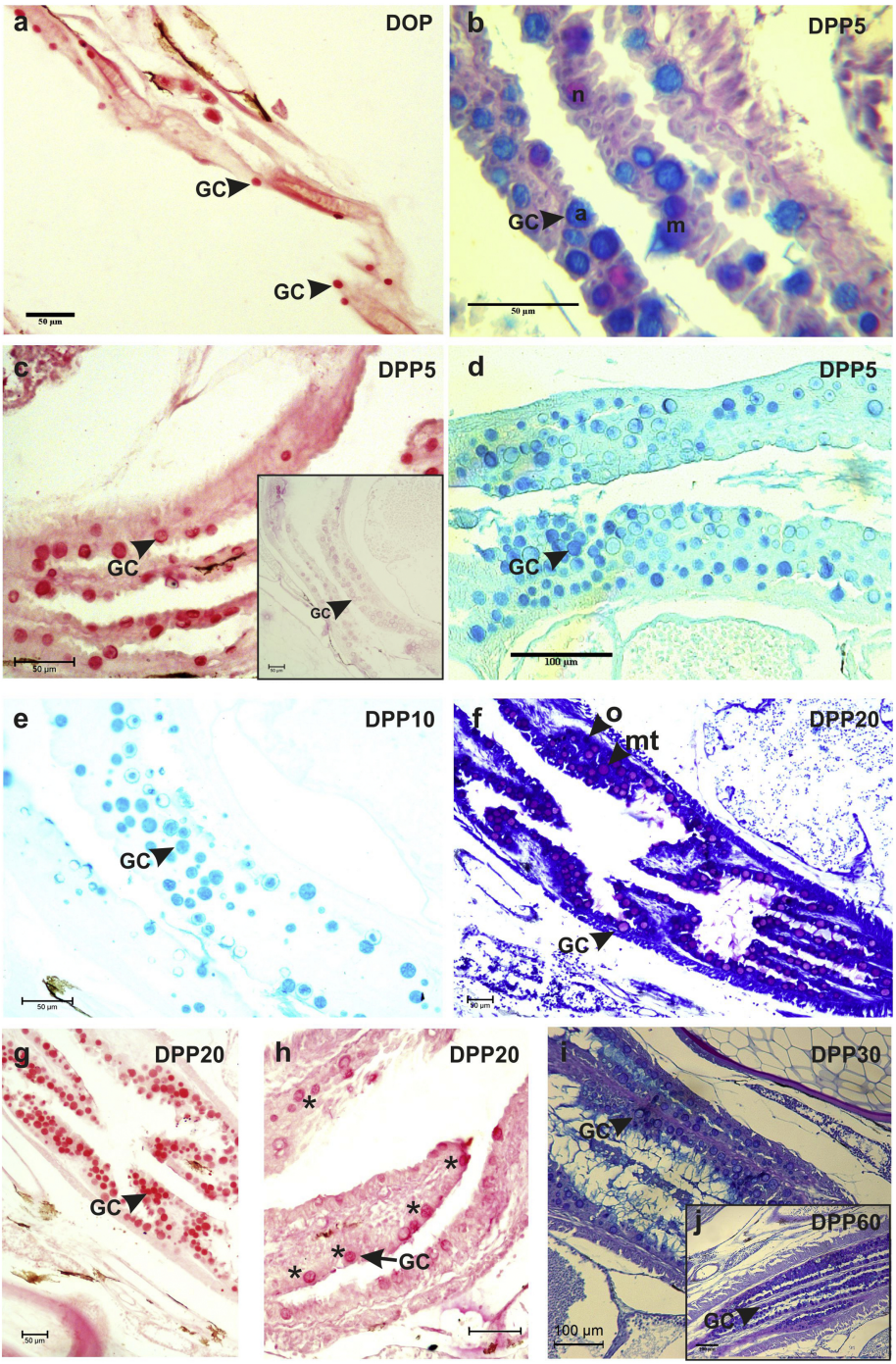
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Figure 1

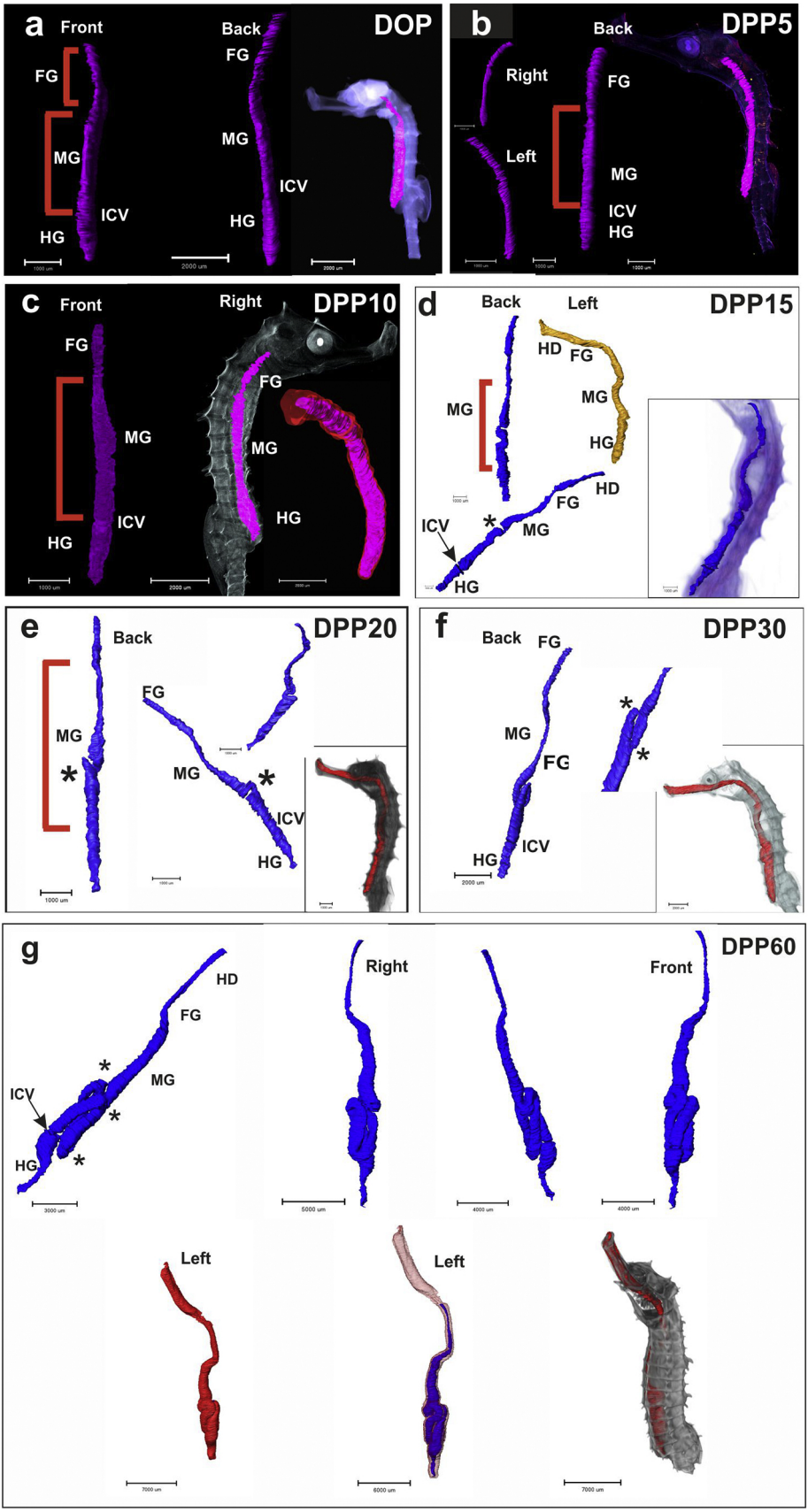
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Figure 2

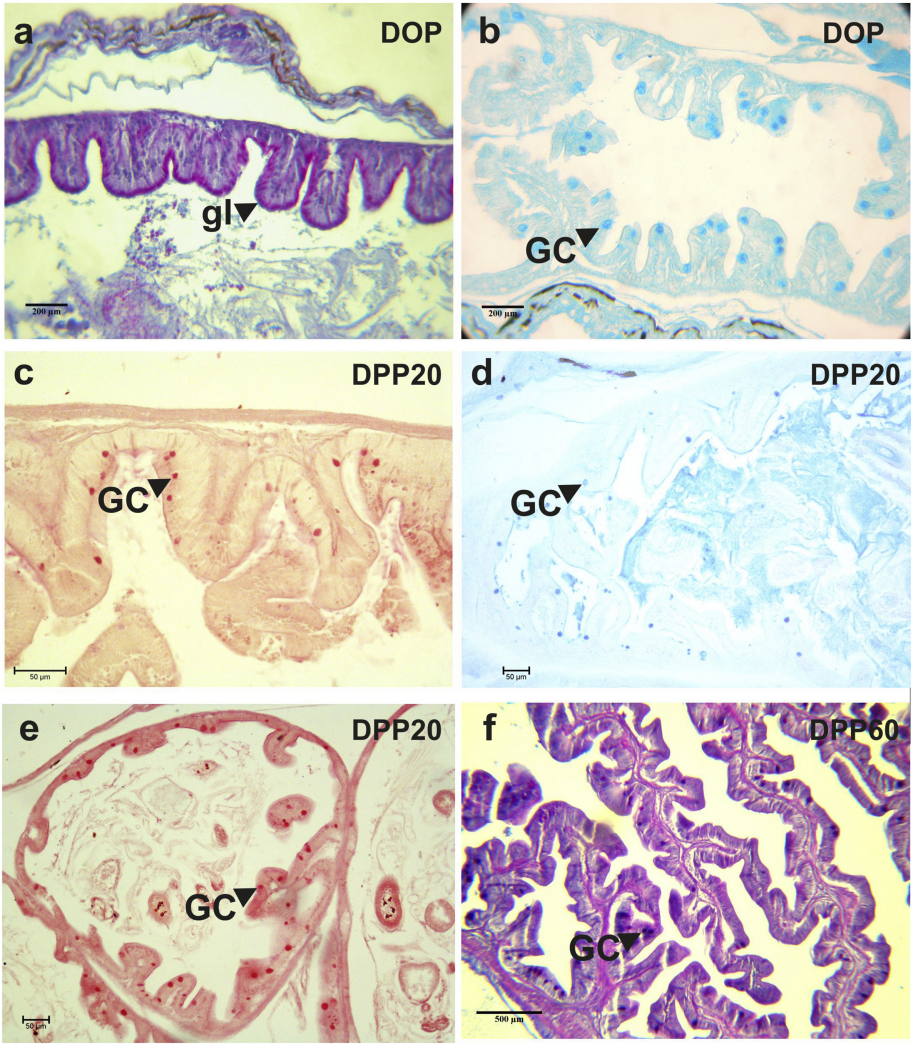
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Figure 3

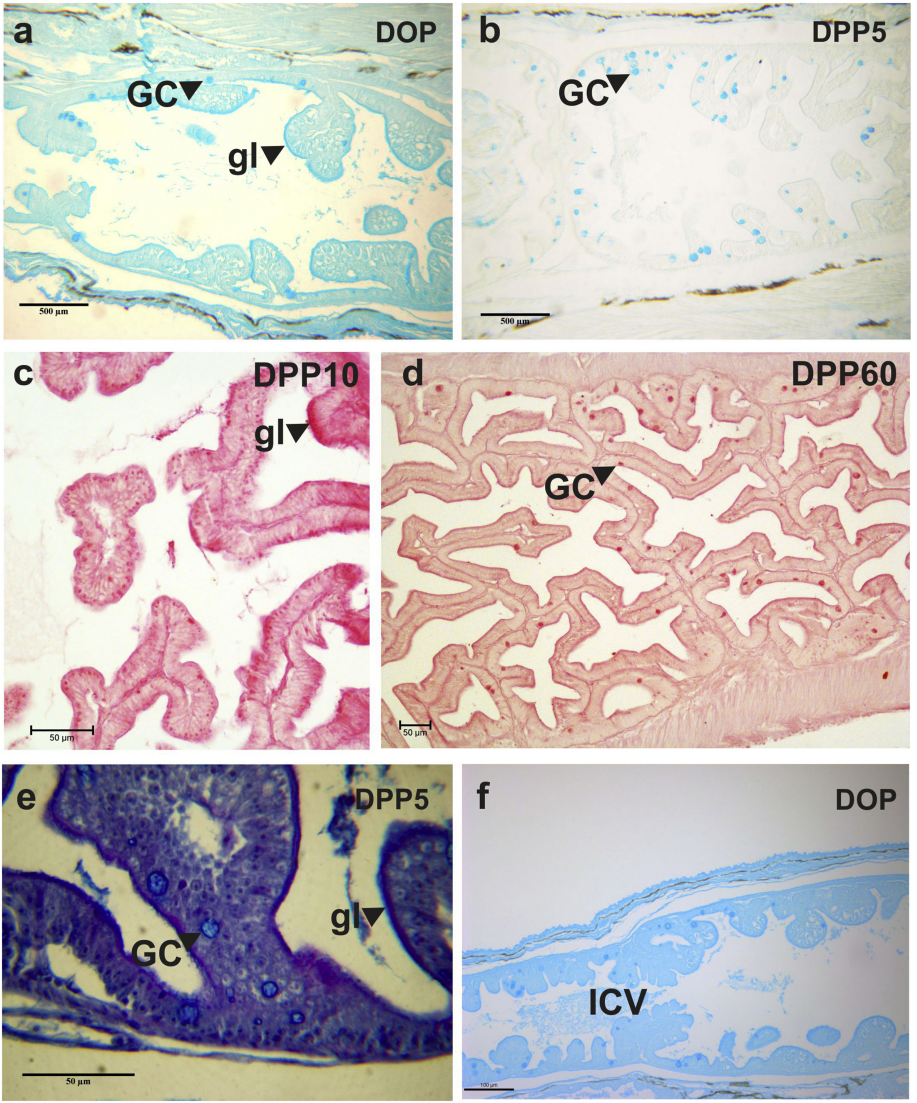
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Figure 4