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Gastrointestinal diversity and function in teleosteans – 2 case studies

Patrícia Gomes Ferreira

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Patrícia Gomes Ferreira

INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

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Orientador – Jonathan Mark Wilson
Categoria – Professor auxiliar
Afiliação – Universidade de Wilfrid Laurier, Waterloo, Canadá

Co-orientador – Luís Filipe C. Castro
Categoria – Investigador auxiliar
Afiliação – CIIMAR (Centro Interdisciplinar de Investigação Marinha e Ambiental)

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To Mother Nature

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Abstract

The stomach is a muscular, sac-like organ, part of the gastrointestinal tract. It is responsible for the secretion of hydrochloric acid (HCl) as well as for the secretion and activation of proteases. The stomach presents a great variability of shapes across vertebrates. Such diversity is the product of an evolutionary response to multiple abiotic (e.g. environmental) and biotic (e.g. diet, competition) pressures. As these pressures fluctuated over time, some organisms appear to have lost the gastric phenotype. Examples can be found in multiple lineages of vertebrates, such as mammals (e.g. monotremes) and numerous fishes (both Chondrichthyes and Osteichthyes) as the chimaeras (Holocephali), the lungfishes (Dipnoi) and a great number of teleosts, including the Tetraodontidae family. This loss of function (or the entire loss of the organ, e.g. Cyprinidae) is intriguing, as the stomach's development is amongst the major innovations that arose with the early gnathostomes. In order to deepen our understanding on this subject, this work aimed to 1) relate the inflation phenotype as a possible driver for gastric function loss in the sargassum fish *Histrio histrio* and 2) test how differences in the stomach morphology correlate with differences in gastric function (i.e. acidification) in three Pleuronectiformes species (*Scophtalmus maximus, Platichtys flesus* and *Solea senegalensis*). As shown in other studies, the gastric phenothype is correlated with the 1) expression of the proton pump H^+/K^+ ATPase and consequent luminal acidification, 2) pepsinogen secretion and 3) secretion of a mucous layer destined to protect the epithelium's surface. Through a combination of histological (Alcian-blue PAS) and imunohistochemical tecnhiques with gene and protein (Western-blot) analysis, it was possible to relate the inflation capability with a full-functional stomach in *Histrio histrio*. This work also allowed me to obtain the first results correlating the morphological gradient in the stomach of the three studied Pleuronectiformes species with differences in the stomach's luminal acidification.

Keywords: stomach loss, gastric proton pump, pepsinogen, stomach acidification

Resumo

O estômago é um orgão musculado em forma de saco, pertencente ao tracto gastrointestinal. É responsável pela secreção de ácido clorídrico (HCl) assim como pela secreção e activação de proteases. O estômago apresenta, nos vertebrados, uma grande variabilidade de formas. Tal diversidade é o producto de uma resposta evolutiva a múltiplas pressões abióticas (ambientais) e bióticas (por exemplo: dieta e competição). Ao longo do tempo, com o decorrer destas pressões, alguns organismos perderam o fenótipo gástrico. Exemplos desta perda podem ser encontrados em múltiplas linhagens de vertebrados como os mamíferos (e.g. monotremata), e numerosos peixes (Chondrichthyes e Osteichthyes). Esta perda de função gástrica em peixes estende-se desde as quimeras (Holocephali), aos peixes pulmonados (Dipnoi) e a um grande número de teleósteos, icluindo a família Tetraodontidae. Esta perda da função (ou perda completa do estômago, e.g. nos Cyprinidae) parece intrigante uma vez que o primeiro aparecimento deste orgão se encontra entre as maiores inovações que emergiram com os primeiros gnatostomados. De forma a aprofundar o conhecimento neste tema, este trabalho teve como objectivos (1) relacionar o fenótipo de inflação como possível causa da perda de função gástrica no peixe *Histrio histrio* e (2) testar o efeito de diferenças na morfologia do estômago na função gástrica (i.e. acidificação) em três espécies de Pleuronectiformes (*Solea senegalensis*, *Plachtichtys flesus* e *Scophtalmus maximus*). Como demonstrado em outros estudos, o fenótipo gástrico está correlacionado com (1) a expressão da bomba de protões H^+/K^+ ATPase, (2) a secreção de pepsinogénios e (3) uma camada mucopolissacárida que protege a superfície do epitélio. Através da combinação de técnicas histológicas (Alcian-blue PAS) e imunohistoquímicas, com análise génica e proteica (Western-blot), foi possível relacionar a capacidade de inflar com o estômago plenamente funcional em *Histrio histrio*. Foi também possível obter os primeiros resultados que correlacionam o gradiente de robustez com diferenças na acidificação luminal no estômago das três espécies de Pleuronectiformes estudadas.

Palavras-chave: perda de estômago, bomba de protões gástrica, pepsinogénio, acidificação do estômago

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List of Acronyms

3APS	3-amino propyltriethoxysilane
APS	Ammonium persulfate solution
ATP4A	H^+/K^+ gene (α subunit)
BSA	Bovine serum albumin
DABCO	1,4-diazabicyclo-[2,2,2]-octane
DAPI	4', 6-diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
GIT	Gastrointestinal tract
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IHC	Immunohistochemistry
LB medium	Lysogeny broth medium
nafl	Net acid flux
PAS	Periodic acid Schiff
PBS	Phosphate buffered saline

PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
PGA	Pepsinogen A gene
PGC	Pepsinogen C gene
ppt	Parts per thousand
SDS	Sodium dodecyl sulfate
SEI	Sucrose, EDTA, Imidazole buffer
SEM	Standard Error of the Mean
tblastx	Translated nucleotide blast
TPBS	Tween-20 in Phosphate buffered saline
TTBS	0.05%Tween-20 in Tris Buffered Saline
uEqH$^+$	Microequivalent of hydrogen ion per litre

Introdutory note

"(...) all of us have in our veins the exact same percentage of salt in our blood that exists in the ocean, and, therefore, we have salt in our blood, in our sweat, in our tears. We are tied to the ocean. And when we go back to the sea - whether it is to sail or to watch it - we are going back from whence we came."

Jonh F.Kennedy, 1962

Chapter 1

General Introduction

"The only law that holds without exception in biology is that exceptions exist for every law"

Stebbins, 1984

1.1 The origin of stomach

It is believed that the stomach's first appearance may be related to the rise of macrophagous feeding (Barrington, 1957). The stomach is a highly specialised digestive organ, characterised by an acid-peptid digestion through the occurrence of hydrochloric acid (HCl) and pepsinogen producing gastric glands. Barrington (1957) suggested a relationship between the appearance of acid secretion and the "need" for prevention of bacterial infections from the ingestion of prey. The author also pointed out that pepsin (which evolved after gastric acidification) appeared as a divergence of intracellular cathepsin.

The emergence of acid and pepsin secreting gastric glands represented an important functional innovation that allowed an extension of dietary choices due to a more efficient breakage of proteins. Although one of the hallmarks of gnathostomes (jawed vertebrates) was the regionalisation of the stomach and its gastric glands, a surprisingly high number of vertebrates lack these structures (figure 1.1; Barrington, 1957; Castro et al., 2014; Pilliet, 1885; Wilson and Castro, 2010).

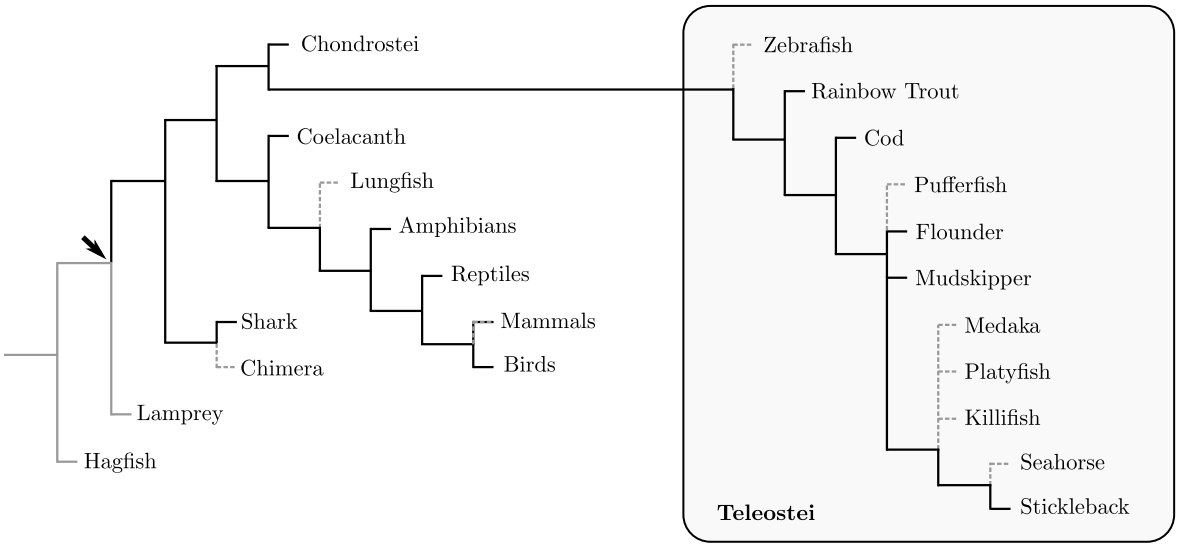


Figure 1.1: Simplified schematic showing the distribution of gastric and agastric phenotypes across different vertebrate groups. The arrow indicates the origin of gnathostomes. Grey lines correspond to agnathans, black (full) lines indicate gastric species/groups, while grey dashed lines point agastric species/groups. The black dashed line at the Mammalia group indicates the presence of a minority of agastric species.

1.2 Fish gastrointestinal physiology

The fish's gastrointestinal tract (GIT) follows a basic organisation, common to many other vertebrates, being divided into four distinct regions: 1) headgut, 2) foregut, 3) midgut and 4) hindgut (Harder, 1975; Horn and Gawlicka, 2001). The fish headgut corresponds to the anterior section of the tract and includes the orobranchial cavity. Its main function is the acquisition and trituration of food (Stevens and Hume, 2004). The foregut corresponds to the esophagus and stomach. In gastric species, the esophagus connects the orobranchial cavity to the stomach and, in species that lack stomach, it directly connects the orobranchial cavity to the intestine. The stomach is highly variable between fish species and is even absent in some groups. This organ serves as a site for storage and initial digestion of food (Horn and Gawlicka, 2001; Stevens and Hume, 2004). Histologically, the beginning of the stomach is well demarcated by the epithelium change from goblet-type to columnar mucous cells. This organ is histologically divided in two regions: cardiac or fundic region (anterior part) and pyloric region (posterior), in the anterior mucosa there can be found gastric or chief glands, which are absent from the pyloric region (Wilson and Castro, 2010). The cardiac zone is known for the presence of gastric glands that secrete pepsinogen and hydrochloric acid (HCl). There is some variability in stomach shape, with a siphonal shape being most common in Osteichtyes and Elasmobranchii, but there are also fish presenting a cecal or even straight (the rarest) stomach shape (Harder, 1975; Pernkopf and Lehner, 1937). The midgut region,

also called the intestine, is responsible for the digestion of carbohydrates, fat and protein across all vertebrates. It is the main site of nutrient absorption, that has high efficiency due to the presence of microvili that increase the luminal surface area. In fish, this organ can present multiple shapes, from short and straight to convoluted and spiral (Wilson and Castro, 2010). In gastric fishes, the intestine may include particular structures to enhance the surface of absorption such as pyloric caeca (Horn and Gawlicka, 2001). The hindgut, as the final portion of the GIT, is sometimes hard to distinguish from the midgut. It is responsible for the recovery of water and electrolytes (Stevens and Hume, 2004). Although the GIT is very similar in organisation across vertebrates, there are some groups that lack some parts or functions, such as the stomach itself and the related acid and pepsinogen activity.

1.3 The gastric glands

The characteristic gastric glands can be found in the anterior part of the stomach, where the columnar epithelium is interspersed with gastric pits. The gastric glands found in fish can be of two types: tubular or acinar (figure 1.2), with the latter being associated with a higher pH environment in the stomach (Wilson and Castro, 2010).

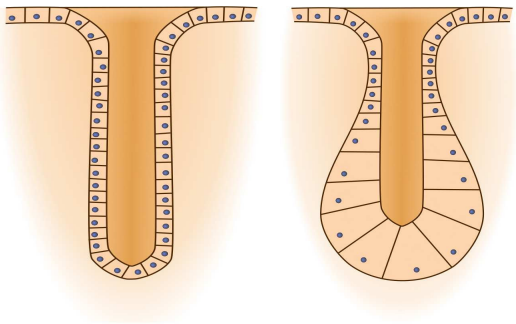


Figure 1.2: Tubular (left) and acinar (right) glands.

In contrast to mammals,which have different cells responsible for the secretion of hydrochloric acid (parietal or oxyntic cells) and for the secretion of pepsinogens (peptic or chief cells), fishes and other vertebrates have a single type of cell responsible for the secretion of both HCl and pepsinogens: the oxynticopeptic cells (Barrington, 1957; Wilson and Castro, 2010).

From a morphological point of view, the secretion of HCl is related to a robust system composed by a tubulovesicular and canalicular network of membranes that allow the active transport of the Cl^- and H^+ into the stomach's lumen (Barrington, 1957).

1.3.1 The gastric pumps

The gastric proton pump, H^+/K^+-ATPase, is a characteristic feature of the gastric glands and is responsible for stomach acidification. It exchanges potassium from the lumen with cytoplasmic H^+. The chloride, transported separately via a Cl^- channel and in conjugation with the H^+, forms HCl (figure 1.3; Sachs et al., 1995), the K^+ diffuses through a K^+ channel. This acid helps in the conversion of pepsinogen to pepsin, a proteolytic enzyme. The conversion also occurs through a process called auto-catalysis, in which the produced pepsin converts pepsinogen into more pepsin, increasing the process' efficiency (Fuentes et al., 2005; Kageyama and Takahashi, 1987). The gastric acidity promotes, in this way, the protein digestion.

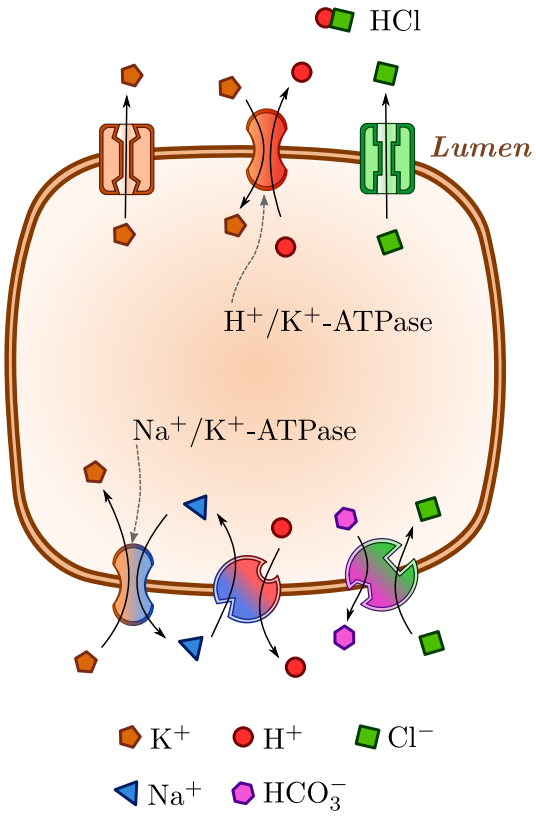


Figure 1.3: Simplified scheme of the acid secretion mechanism: The H^+/K^+-ATPase in the luminal side of the oxynticopeptic cells takes H^+ ions into the lumen in exchange for K^+. For every H^+ ion secreted, one HCO_3^- is exchanged for a Cl^-. Cl^- ions enter the lumen through a Cl^- channel (adapted from Barrett et al., 2006).

At the molecular level, the H^+/K^+-ATPase is a heterodimeric protein that appears as the combined product of two genes, *ATP4A* and *ATP4B*. The *ATP4A* gene encodes the α subunit, containing the catalytic sites of the enzyme. This subunit forms a pore in the cell membrane through which the ions are transported. The *ATP4B* gene encodes the β subunit, responsible for the stabilisation of the α subunit and for the maintenance of the normal running of the process, its heavy glycosylation it is important in the protection from acid and proteolytic damage in the lumen, (mutants for this β subunit were found incapable of prevent reverse running of the enzyme; Abe et al., 2009).

The acidic secretion of this pump when released into the stomach lumen may be harmfull for the epithelial cells. To prevent this acid damage, an alkaline barrier of glycoproteins, mucins, is formed. This mucus also prevents the pepsin to reach the epithelial surface and its consequent proteolysis (Perez-Vilar, 2007).

1.3.2 Pepsinogens

Pepsin is the major digestive enzyme in the animal's stomach. It is secreted as the inactive pro-enzyme, pepsinogen, by chief cells of oxyntic glands in mammals and from oxynticopeptic cells in fishes and other vertebrates. In the acidic environment of the lumen of the glands, pepsinogen rapidly converts to pepsin through a change in its conformation that involves a cleavage of a prosegment at the N-terminus of the zymogen (i.e. pepsinogen; Kageyama, 2002; Richter et al., 1998). Five types of pepsinogens are known: pepsinogens A (*PGA*), B (*PGB*), F (*PGF*), progastricsin (*PGC*), and prochymosin (*CYM*). From a molecular evolutionary perspective, it is believed that the first group of pepsinogens to diverge was the *PGC* family and the last ones the *PGA* and *PGF* families, that are the two most related (Kageyama, 2002). The phylogeny of pepsinogens is considered to be useful for estimating phylogenetic relations within vertebrate groups. Particularly, the pepsinogen C, due to its low number of different copies, as been indicated as a worthy molecular marker (Castro et al., 2012).

Histologically, pepsinogen can be identified as eosinophilic zymogen granules (Wilson and Castro, 2010).

1.4 Agastric fishes

Although GIT organisation and function is similar across the majority of the vertebrates (Barrington, 1957), some groups are known for lacking the stomach glands, or even the entire stomach (Kurokawa et al., 2005; Pilliet, 1885; Wilson and Castro, 2010), where the esophagus connects directly to the intestine. This lack of stomach is known in several fish groups, such as the Cypriniformes, Beloniformes, and Tetraodontiformes and in non-teleosts as holocephali, dipnoids and monotremes (Castro et al., 2014; Wilson and Castro, 2010).

1.4.1 Potential drivers of stomach loss: The inflation phenotype

Many hypotheses have been pointed out in an attempt to understand the drivers for stomach loss events. This includes the high cost of acid-peptic digestion (that brings also a need for protective mucins production), the diet or even the buffer capacity of the water in the case of aquatic organisms that are present in environments with low levels of Cl^- (Bergman et al.,

2003; Horn et al., 2006; Lobel, 1981; Wilson and Castro, 2010). Nevertheless, there is still a need for further research addressing this thematic in a broader perspective, in order to understand the multiple stomach loss events that happened during the course of evolution, after the first appearance of this organ with the early gnathostomes.

The pufferfishes family, Tetraodontidae, is a curious case of stomach loss. This tetraodontiformes are known for their ability to inflate as a defence response, and this mechanism seems to have displaced the gastric function in this species since no gastric glands nor the expression of the proton pump H^+/K^+-ATPase or pepsinogens have been detected in it (Brainerd, 1994; Castro et al., 2014; Kurokawa et al., 2005).

The inflation process, usually associated with the members of Tetraodontidae and Diodontidae families (Order Tetraoditonformes), presents a major evolutionary feature, as it allowed for an increased defence capability against larger predators (Brainerd, 1994). Diverse physical characteristics enable inflation in this group, with the highly elastic skin in the lateral side of the body standing out (Wainwright and Turingan, 1997).

In his work, Wainwright and Turingan (1997) identified three different mechanisms: 1) coughing, an expulsion of unwanted materials over the mouth; 2) water blowing, an action that combined an uptake of water into the mouth, followed by an ejection of this water through the mouth and 3) inflation, characterised by the ability to retain pumped water in the stomach, leading to an increasing in the fish's body volume.

This inflation allows the fish to dissuade a potential predator's attack. Wainwright and Turingan (1997) also reported the presence of water-blowing behaviour in several families: Balistidae, Monocanthidae, Triodontidae, Molidae and Ostraciidae, while coughing is visible in almost all fishes. Despite the author's limitation of the inflation behaviour only to the Diodontidae and Tetraodontidae (both from the Tetraodontoformes), more fishes are known for their ability to inflate their body, as it is the case of the swell shark, *Cephaloscyllium ventriosum* (Clark, 1947), and the sargassum fish, *Histrio histrio* (Gordon, 1938; Pietsch and Grobecker, 1987). *Histrio histrio*'s case will be focused on in this work.

1.5 Statement of dissertation objectives

The proposed goals of this dissertation were to explore: 1) inflation as a possible driver for, or a result of, stomach loss in teleosteans, through a molecular analysis of the sargassum fish

Histrio histrio; 2) how the diversity of stomach morphology can influence the physiological function of acidification, using three species of Pleuronectiformes, *Scophtalmus maximus, Plathychtys flesus* and *Solea senegalensis* as study models.

This dissertation is organised into four chapters, starting with a *General Introduction* (Chapter 1), Chapters 2 and 3 are experimental work, *Histrio histrio – Inflation and gastric function* and *Diversity and function of Pleuronectiformes Gastrointestinal tract*, respectively, structured as papers and finishing with *Final Considerations and Future Perspectives* (Chapter 4).

Chapter 2

Histrio histrio – Inflation and gastric function

2.1 Abstract

The sargassum fish *Histrio histrio* is a marine fish that is found usually associated with *Sargassum* sea weed. Although it is a poorly studied species, it has been reported that this species has the capacity to inflate its body when threatened. The aim of this study was to determine if this inflation capacity was associated with a loss of stomach function - the presence of active gastric glands that secrete HCl and pepsinogen - as seems to have happened in some tetraodontiforme fishes. The stomach was confirmed by histology and imunohistochemistry that detected the gastric proton pump H^+/K^+-ATPase. In addition, through molecular methods it was possible to identify and sequence diverse genes associated with the gastric function: *atp4a* (the α subunit of the H^+/K^+ proton pump , and two pepsinogens *pgc* (pepsinogen C) and *pga* (pepsinogen A). Protein expression was also confirmed through Western blotting. Taken together, these results indicate a fully functional stomach in *H. histrio*, demonstrating the possibility of coexistence of both gastric and inflation phenotypes.

2.2 Introduction

2.2.1 *Histrio histrio*

The Lophiiformes order includes a great number of species from 18 marine families, amongst which are the deep-sea fishes from the *Melanocetidae* family, the sea toads, *Chaunacidae*, and the frogfishes, representatives of *Antennariidae* family (Shedlock et al., 2004). The frogfishes are typically small-sized, with bulbous forms and, in similarity with the other Lophiiformes, they have an extension of the first dorsal spine on the dorsal fin (*illicium*) that is projected forward and acts as a lure for prey (Pietsch, 1984; Shedlock et al., 2004).

Within the *Antennaridae* family there is a particular species that inhabits the sargassum complex and that it is known by its territoriality and voracity, the sargassum fish *Histrio histrio*. *H. histrio* has a smooth skin that presents numerous cutaneous projetions (that serve as camouflage in the sargassum where it usually inhabits), a small *illicium* and jointed leglike pectoral and pelvic fins (Pietsch, 1984), see figure 2.1.

Histrio histrio has a cosmopolitan distribution in tropical and subtropical seas where it can be found until 10 metres depth. It is an epipelagic species, and the only one within its family that is usually found associated to floating *Sargassum* (McEachran et al., 2015; Pietsch and Grobecker, 1987).

(a) (b)

Figure 2.1: **(a)** *Histrio histrio* in maintenance aquarium; **(b)** plastic strips were used to simulate sargassum seaweed during the maintenance period.

2.2.1.1 *Histrio*'s inflation

The sargassum fish is widely known as a highly territorial and voracious species, capable of eating preys bigger that itself (Gordon, 1938; Pietsch and Grobecker, 1987). But it is not for the remarkable attack capacities that this fish is surprising. Several authors have commented, through the past centuries, the frogfish's ability to inflate (Gordon, 1938; Pietsch and Grobecker, 1987), i.e. the capability to suddenly intake a considerable amount of water into its stomach and, through this action, expand quickly its body volume, much like the pufferfishes (Wainwright and Turingan, 1997). This mechanism is believed to be used as a way to avoid potential predation, as they are too large to swallow.

2.2.2 Stomach's diversity

The stomach is the first site of digestion in the gastrointestinal tract (GIT), known by the its glands and associated proton pump (the H^+/K^+-ATPase) responsible for the secretion of hydrochloric acid (HCl) and pepsinogen (Wilson and Castro, 2010). Despite being a common feature in gnathostomes' GIT, several vertebrate groups lack a stomach (Castro et al., 2014; Wilson and Castro, 2010). This absence in vertebrate lineages is particularly common amongst teleosteans. Many pressures have been pointed out as potential drivers for stomach loss; such as the diet, the salt content of the water in which the animal inhabits or even the replacement of the gastric function for another (Bergman et al., 2003; Horn and Gawlicka, 2001; Kurokawa et al., 2005; Wilson and Castro, 2010). An example of this stomach function replacement can be seen in the pufferfishes, where the stomach lacks any glandular activity and has a defence function (i.e. inflation; Brainerd, 1994; Castro et al., 2014; Kurokawa et al., 2005).

2.3 Objectives

This work aimed to reveal if, in parallel to the pufferfish, the inflation ability reported for *H. histrio* is accompanied by the loss of stomach function, i.e. the loss of stomach acidification and pepsinogen secretion related to the absence of secreting glands. It is expected that through the validation or refutation of such hypothesis, new light can be shed on the evolutionary process of stomach loss.

2.4 Material and methods

2.4.1 Animals

Adult sargassum fish, *Histrio histrio*, were purchased from Tropical Marine Centre fish supplier. Upon arrival fish were maintained in 50 L glass aquaria containing aerated salt water(33 ppt) at 25℃, under a controled photoperiod (12^L /12^D hours) and fed daily until 48 hours prior to sampling.

2.4.2 Sampling

Fish were euthanized with an overdose of neutralized tricaine methane sulphonate, MS-222 (1:5000 (w/v)), followed by a cervical transection. Organs (stomach, gills, liver, brain, eye, kidney, intestine, spleen, skin and muscle) were collected both for RNA and protein extraction by immediately freezing with liquid nitrogen and to histology/immunohistochemistry analysis, by immersion in fixative, 4% paraformaldehyde/phosphate buffered saline (PFA/PBS) pH 7.4, for 24 hours at 4℃.

2.4.3 Histology

The tissues were progressively dehydrated through an ascending ethanol series (70%, 95% and 100%), cleared in xylene and finally embedded in paraffin. Sections were cut at 5 μm with a Reichert Biocut 2030 microtome and stained with Alcian-blue PAS (Periodic acid-Schiff) and hematoxylin. Sections from the entire length of the stomach were observed and images were acquired with a Leica DFC300FX digital colour camera mounted on a Leica DM 6000 B microscope.

2.4.4 Immunohistochemistry

Paraffin sections (5μm) were collected onto 3APS (3-amino propyltriethoxysilane; Sigma-Aldrich, St Louis, MO) coated slides and allowed to air dry. After drying, samples were dewaxed through xylene and ethanol and the sections were circled with a hydrophobic barrier (PAP pen, Sigma-Aldrich). Samples were then blocked with 1% BSA/TPBS (0.05% Tween-20 in Phosphate Buffered Saline (137 mM NaCl, 2.7 mM KCl, 7.8 mM Na_2HPO_4), pH 7.4) for 20

minutes at room temperature. Sections were incubated with a combination of the antibodies T4 (mouse monoclonal) that detects the Na^+:K^+:Cl^- co-transporter activity (Ip et al., 2013) and C2 (rabbit polyclonal) that detects the H^+/K^+-ATPase (Smolka and Swiger, 1992), diluted respectively at 1:100 and 1:200 in 1% BSA/TPBS, overnight at 4℃ in a humidified chamber. The negative control sections were incubated only in BSA/TPBS. After the incubation, slides were washed with TPBS for 5, 10 and 15 minutes and incubated with goat anti-mouse Alexa Fluor 594 and goat anti-rabbit Alexa Fluor 488 conjugated secondary antibody, both diluted 1:500 (Molecular probes Inc, Eugene, OR) in BSA/TPBS for 1 hour at 37℃. After secondary-antibody incubation, slides were washed again with TPBS and nuclei were then stained with DAPI (4', 6-diamidino-2-phenylindole, SigmaAldrich) during the 10 minute rinse. Coverslips were mounted using 1:1 PBS glycerol. The sections were observed on a Leica DM6000 B wide field epi-fluorescence microscope and the images were captured using a cooled digital camera (Leica DFC 340 FX).

2.4.5 SDS-PAGE and Immunoblotting (IB)

For obtaining a protein expression tissue profile, pools of samples (3 individuals per pool) from gill, stomach, spleen, kidney and liver were treated as follows. Samples were sonicated for 2x 10 seconds (Sonics & Materials Inc., Newtown, CT, USA) in 250 μL SEI buffer (300 mmol sucrose, 20 mmol EDTA, 100 mmol imidazole, pH 7.3). The homogenates were centrifuged at 14,100 rcf (relative centrifugal force) for 2 minutes (Eppendorf AG, Mini Spin plus, Hamburg, Germany). Supernatants were decanted and aliquots conserved at 4 ℃ to measure total protein concentration. The remaining supernatant was diluted with an equal volume of 2x Laemmli buffer (0.125 M Tris-HCl, pH6.8, 20% Glycerol, 4% SDS, 0.01% Bromophenol Blue, 100 mM DTT (Dithiothreitol)). This mixture was heated to 70℃ for 10 minutes and stored at 4℃.

Protein was measured using the Bradford dye binding assay (BioRad) with a bovine serum albumin (BSA) standard. The protein concentrations of the samples in Laemmli's buffer were adjusted to 1 μg/μL using 1x Laemmli's buffer in order to have uniform loading volumes.

Thirty μg of protein from each sample were separated by polyacrylamide gels electrophoresis on 10% resolving gels with 4% stacking gels, using the BioRad MiniProtean III system (BioRad). The separated protein bands were then transferred to PVDF membranes (Amersham , GE Healthcare) using a semi-dry transfer apparatus at 13 V for 1 hour (BioRad). After

rinsing the membranes in TTBS (0.05%Tween-20 in Tris Buffered Saline (Tris Base, 0.02 mM Tris HCl, 500 mM NaCl), pH 7.4), they were blocked with 5% powered skim milk in TTBS for 1 hour and briefly rinsed in TTBS before probing with the antibodies: C2 (rabbit primary antibody), for the detection of H^+/K^+-ATPase α subunit at 1:10 000 and 12G10 (mouse monoclonal primary antibody) antibody that detects the reference protein: α-tubulin, at 1:500, diluted in 1% BSA/TTBS overnight at room temperature. After the primary incubation, membranes were rinsed in TTBS and incubated, for 1 hour at room temperature, with goat anti-mouse (1:50 000) or goat anti-rabbit (1:100 000) HRP conjugated IgG secondary antibody, diluted in TTBS. Following the secondary incubation, membranes were rinsed in TTBS before signal detection by enhanced chemiluminescence, ECL reagents, (Immobilon Millipore, Madrid, Spain) using a Fujifilm LAS 4000 mini system.

2.4.6 RNA isolation and cDNA synthesis

Tissues were homogenized using a tissue homogenizer, *Precellys®24* (Bertin Technologies, France) and RNA isolated using the Aurum Total RNA Mini Kit, with on-column DNAse I treatment, following manufacturer's instructions (Bio-Rad, Hercule, CA. Concentration and purity of total RNA was assessed by nanodrop UV spectroscopy at 260/280 nm wavelength and integrity by electrophoresis in a 1.2% formaldehyde agarose gel. Samples were then stored at -80℃ until use. The cDNA was synthesized from 1 μg of total RNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystem, Foster City, CA), according to manufacturer's instructions, and stored at -20℃.

2.4.7 Gene identification and sequencing

After cDNA synthesis, a PCR for the detection of the house-keeping gene, β-actin was performed using Phusion Flash High-Fidelity PCR Master Mix (Finnzymes, Espoo, Finland) ^{TM} enzyme mix , 10 μL, 7.2 μL H_2, 1 μL of each (Reverse and Forward) primer 0.8 μL of sample cDNA . The conditions for this semi-quantitative analysis were a first cycle of 10 seconds at 98℃, followed by 39 cycles of denaturing 1 second at 98℃, annealing 5 seconds at 60℃ and elongation, 30 seconds at 72℃, after the 39 cycles, a there was a final elongation at 72℃ for 2 minutes. The β actin primers were originally designed by Santos et al. (1997) for *Sparus aurata*. Oligonucleotide primers' sequences used in this study are presented in table 2.1.

For the detection of the *atp4a* gene, degenerated primers designed by (Choe et al., 2004) were used with the following amplification parameters: a initial denaturation at 98℃ for 10 seconds, followed by 45 cycles of 1 second at 98℃, 5 seconds at 52℃, 30 seconds at 72℃ and a final 2 minutes at 72℃ elongation. PCR for the *pga* and *pgc* detection were as follows, *pga*: a first cycle of 98℃ for 10 seconds, followed by 45 cycles of 1 second at 98℃, 5 seconds at 58℃, 30 seconds at 72℃ and a final 2 minutes at 72℃ elongation; *pgc*: 10 seconds at 98℃, 32 cycles of 1 second at 98℃, 5 seconds at 53 ℃, 45 seconds at 72℃, and 2 minutes at 72℃.

PCR was done for all primer combinations with 0.8 μL of sample cDNA, 10 μL Phusion Flash High-Fidelity PCR Master Mix (Finnzymes, Espoo, Finland) ^{TM} enzyme mix, 7.2 μL H_2O and 1 μL of each (Reverse and Forward) primer.

The PCR products were loaded, 2 μL of each reaction, onto a 2% agarose gel, with 1:10 000 GelRed^{TM} for the DNA staining, and ran at 80 V for 30 minutes. Gel images were acquired with a Fugifilm LAS 4000 mini system. Post image treatment, brightness and contrast only, adjustments were made, mantaining always the full integrity of the data.

PCR product was purified using Illustra GFX PCR DNA Purification Kit (GE Healthcare) and directly sequenced (StabVida, Caparica, Portugal).

For the sequencing of *pgc* gene it was necessary to clone the PCR amplification product of of this gene. The clonning procedure was conducted as follows.

Table 2.1: Oligonucleotide primers used in this study

Primer	Sequence 5'-3'	Author
β-actin F	GGCCGCGACCTACAGACTAC	Santos et al. (1997)
β-actin R	ACCGAGGAAGGATGGCTGGAA	Santos et al. (1997)
ATP4A F	GAYGARCARTGGAARGARGC	Choe et al. (2004)
ATP4A R	GGRAACCANCCYTCYTGNGCC	Choe et al. (2004)
PgA F	TGACCGGCATCCTGGGNTAYGANAC	*Filipe Castro, unpublished*
PgA R	GCCCCTGGTTCATCATGTTRTCRAANAC	*Filipe Castro, unpublished*
PgC F	GCTSWCKYTYGGAGRVGTGGA	Xue et al. (2013)
PgC R	RGRSGGCAGGTAKGTGGG	Xue et al. (2013)

2.4.7.1 Cloning

After the PCR product purification, Illustra GFX PCR DNA Purification Kit (GE Healthcare), the DNA fragments were included in a cloning vector using the kit *pCRTM8/GW/TOPO® TA Cloning® Kit* (Invitrogen), a schematic of the vector is presented in figure 2.2.

The product of the ligation reaction was incorporate in competent bacterias *One Shot® Chemically Competent E. coli*: 1 μL of plasmid was added to 50 μL of competent bacteria, followed by an incubation in ice for 30 minutes, a heat shock at 42℃ for 30 seconds followed by 2 minutes in ice.

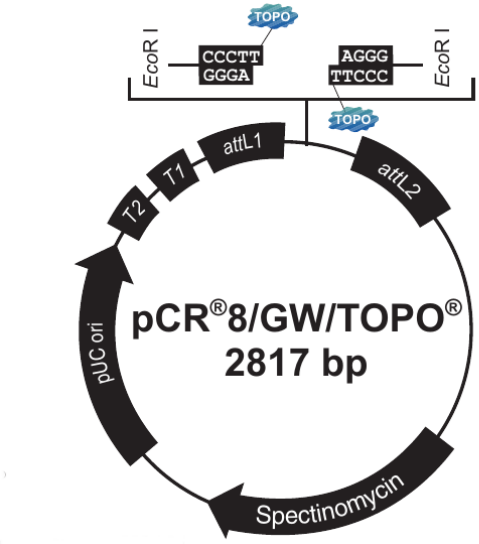


Figure 2.2: Scheme of the pCR^{T}M8/GW/TOPO vector.

Following the transformation, bacteria were left to grow in LB medium at 37℃ for one hour in a shaking incubator. After this step the transformed bacteria were spread on prewarmed LB-agar plates, (30 μL per plate) with Spectinomycin.

The plates were incubated for 16 hours at 37℃. After this incubation the white colonies were selected and allowed to grow individualy in 10 mL of LB medium with Spectinomycin, over-night at 37℃.

After this second incubation the plasmid DNA was isolated with the *PureLink® Quick Plasmid Miniprep Kit* (Invitrogen). Plasmids were then linearized through an enzymatic hydrolise with a restriction endonuclease, EcoRI, for 1 hour at 37℃. The success of the linearization was confirmed through an electrophoresis and the product was then sequenced.

2.4.7.2 Phylogenetics

The retrieved results from sequencing were analysed through a translated nucleotide blast, tblastx (NCBI), and confirmed as the requested gene. Sequences from each gene (*atp4a, pgc, pga* were translated to amino acids in *ExPASy* proteomics Web server. The sequence data for the phylogenetic analysis were obtained from GenBank and Ensembl. Protein sequence alignments and Neighbor-joining trees with bootstrap analysis were generated with Geneious

(v. 9.1.5; http://www.geneious.com; Kearse et al., 2012). The phylogenetic analysis were performed using partial protein sequence.

2.5 Results

2.5.1 Gastric glands identification and proton pump detection by IHC

The sections obtained from *Histrio*'s stomach and stained with Alcian-blue PAS show the presence of tubular gastric glands figure (2.3 d). It is possible to distinguish a neutral magenta staining layer through all the surface of the sections and inside the gastric glands - the basic mucins secreted by the neck cells inside the gastric glands and foveolar cells present at the surface epithelium, figure 2.3 a), b), c). Due to residues of eosin in the stain solutions, it was possible to acquire images of the zymogen (pepsinogen) granules that are visible in the gastric glands, figure 2.3 e).

The immunohistochemistry allowed for the first detection of H^+/K^+-ATPase pump. A strong antibody reactivity against this proton pump is visible across the gastric glands. The presence of Na^+:K^+: Cl^- co-transporter is also visible in a lateral position to the H^+/ K^+ pump, figure 2.4.

2.5.2 *ATP4A* and pepsinogen expression

Through PCR and Western-blot analyses it was also possible to identify, the expression of the *atp4a* gene and the protein activity, see figure 2.5. The α-tubulin protein was used as positive control across the tested samples: gill, stomach, kidney, spleen and liver and immunoreactivity is visible for this protein across the tissue profile. Reactivity against the C2 antibody (H^+/K^+-ATPase) is high in stomach sample, additionally, positive reaction is visible in both gill and kidney samples. Primer pairs ATP4AF and ATP4AR resulted in a single band for *Histrio histrio* stomach with 1000 bp. Sequencing followed by tblastn (NCBI) sequence analysis retrieved a value of 91% of identity to the H^+/K^+-ATPase alpha subunit in *Siniperca scherzeri*. Pepsinogens, *pga* and *pgc*, were identified by degenerated primer PCR, purified and sequenced, retrieving sequences of 325 bp and 402 bp, respectively. Blast analysis result in 85% identity to *Siniperca chuatsi* pepsinogen A1, for the sequenced *pga* and in 79% identity to *Trematomus bernacchii* gastricsin for the sequenced *pgc*. The alignments resultant

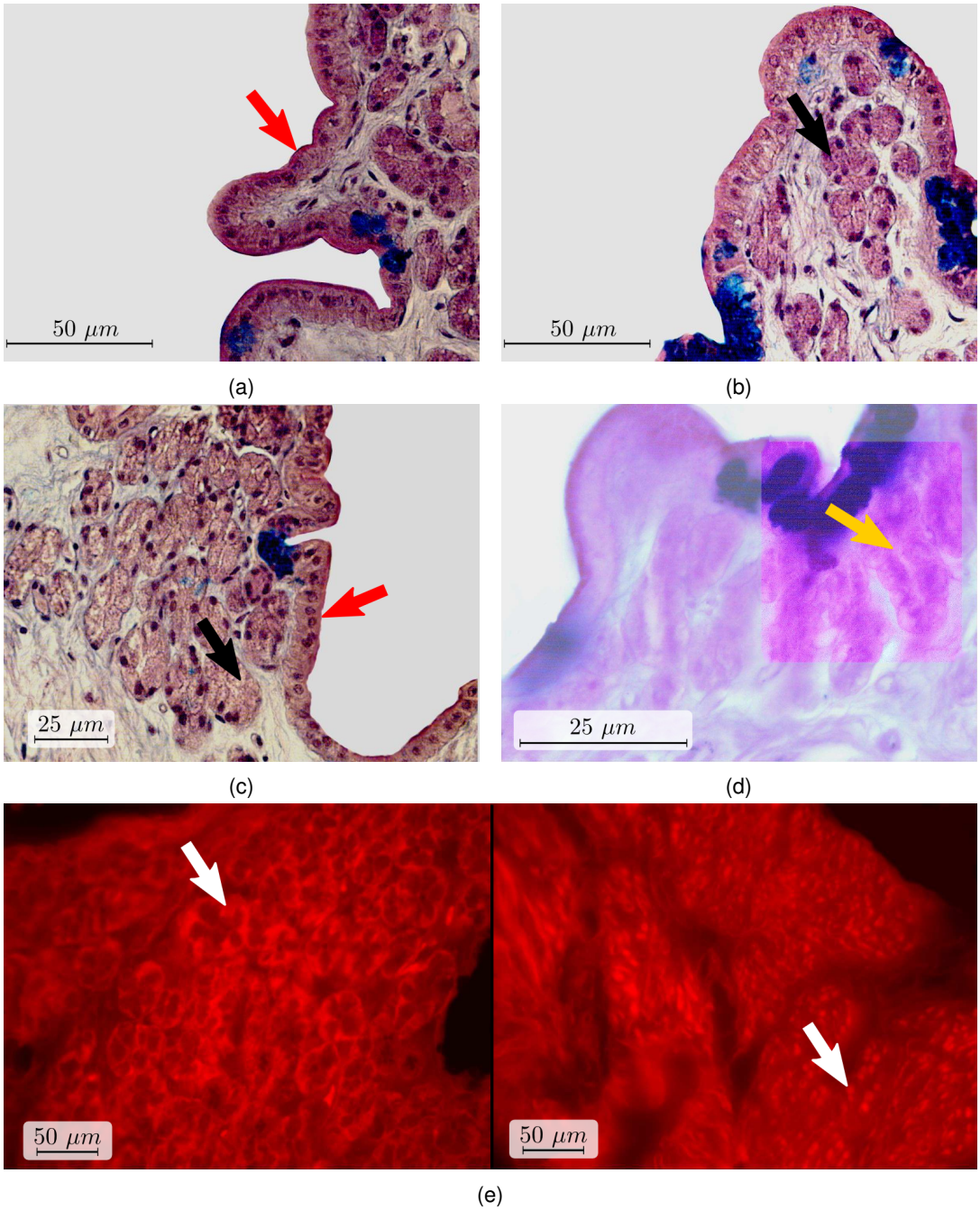


Figure 2.3: Histology results for *Histrio histrio* showing the presence of gastric glands: **(a)**, **(b)** and **(c)** show a epithelial mucous layer (red arrows) and glandular mucous (black arrows); **(d)** shows a longitudinal section of a tubular gastric gland (yellow arrow); **(e)** shows zymogen granules in lighter red (white arrows).

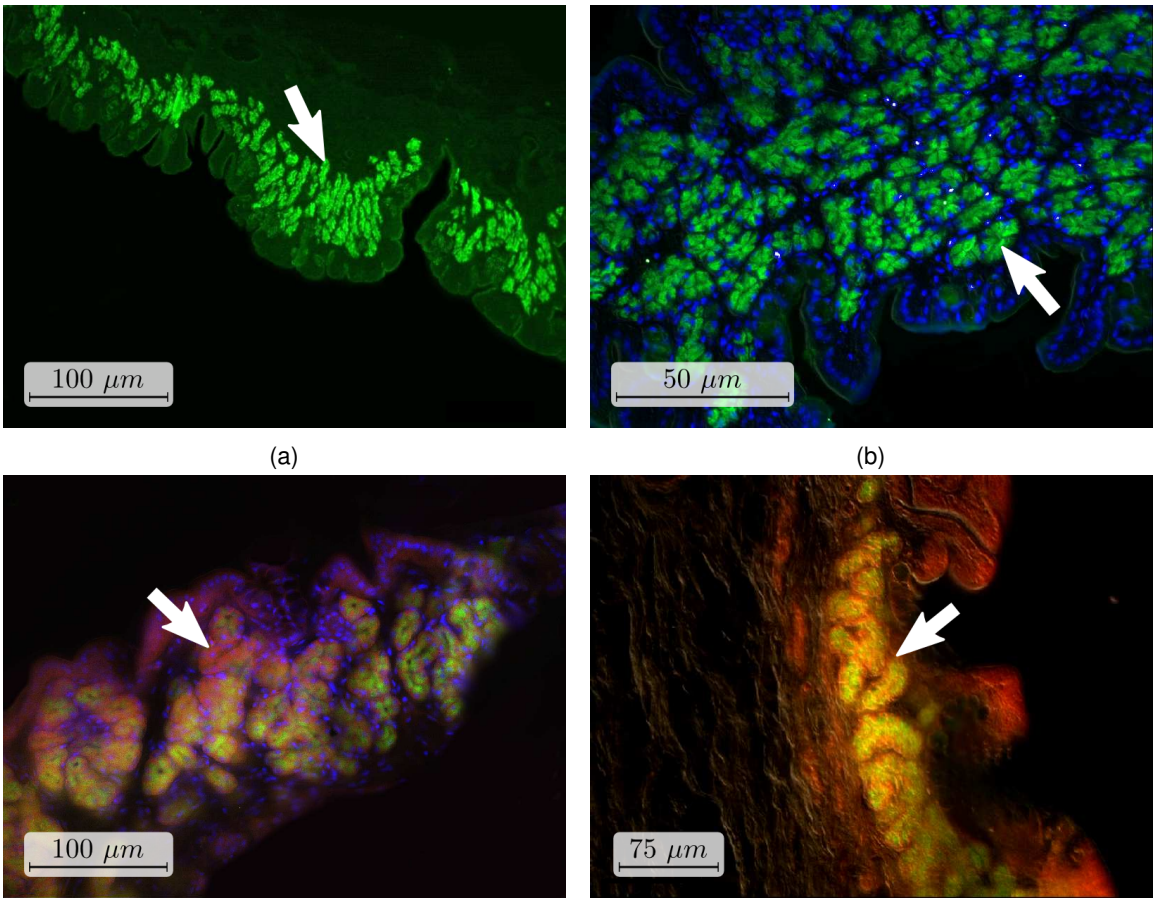
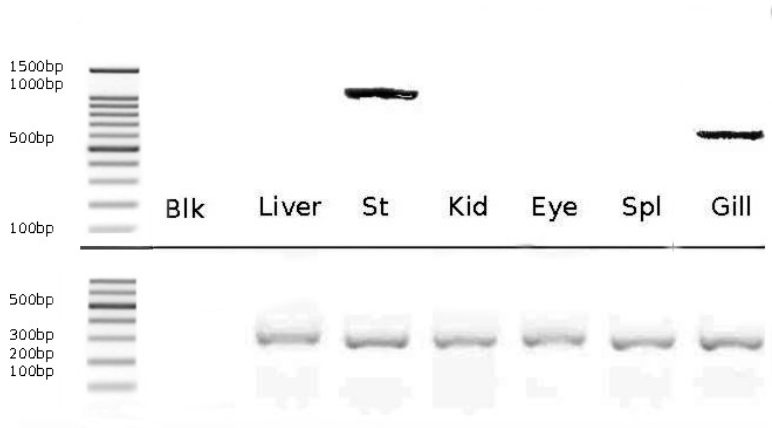


Figure 2.4: Imunostaining sections from *Histrio histrio* stomach: **(a)** and **(b)** show the H^+/K^+-ATPase in green (white arrows); **(b)** also shows nuclear DNA stained in dark blue with DAPI; **(c)** and **(d)** show the Na^+/K^+: Cl^- co-transporter in pink/red (white arrow).

(a)



(b)

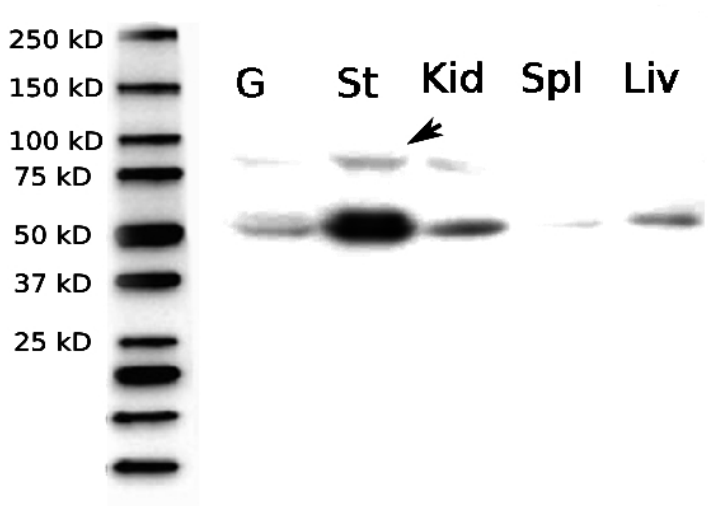
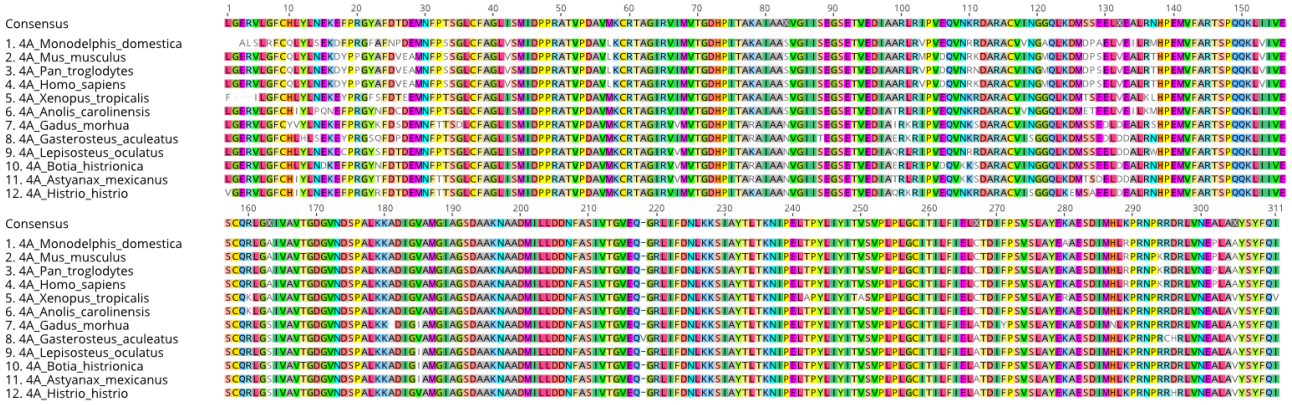
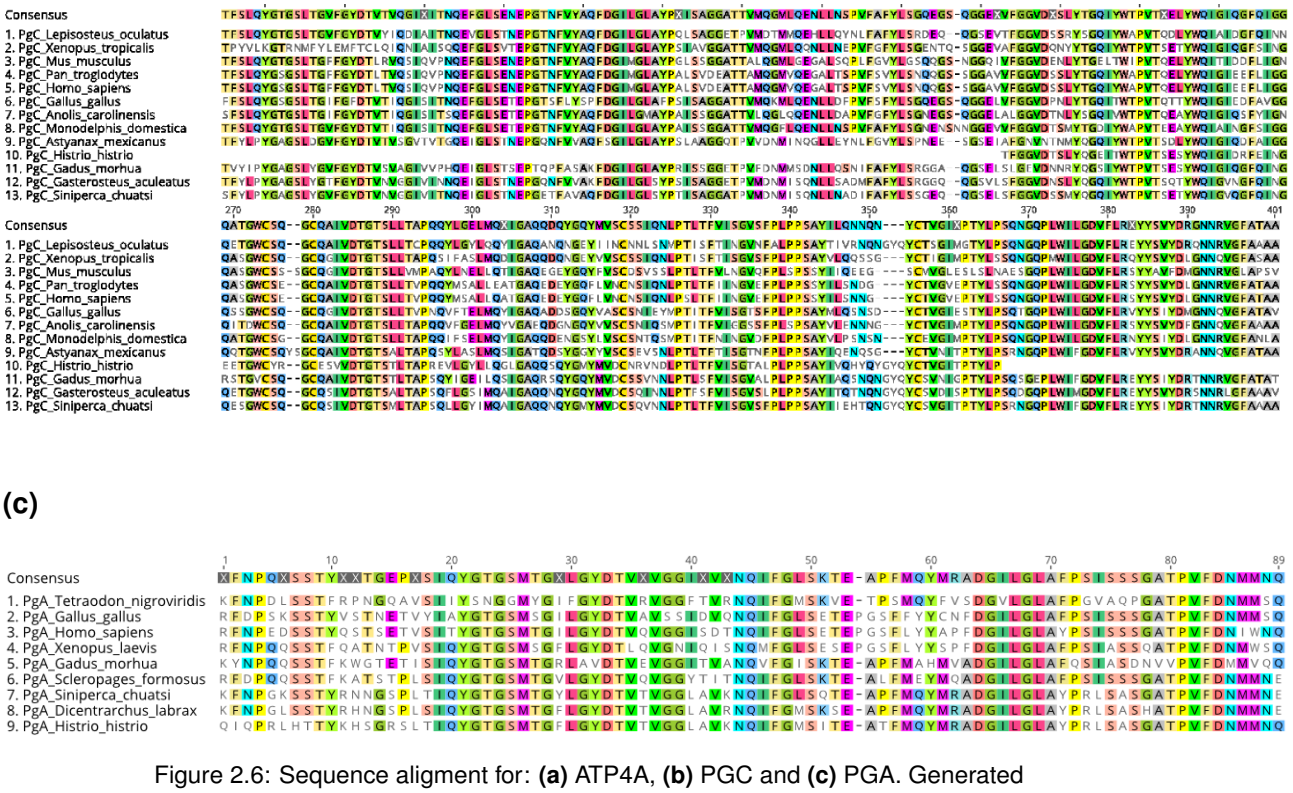


Figure 2.5: *ATP4A* gene and protein expression: the upper part of **(a)** shows the *atp4a* gene detection in stomach (1000 bp) and gills (500 bp) after a PCR with degenerated primers, while the below part shows the PCR result for the house keeping gene β-actin (250 bp); **(b)** shows the Atp4a protein expression detection in stomach and gills (100 kD) and also the reference protein α-tubulin expression across every tissue used (50 kD). The ladders were annexed for size results reference. Blk = Blank; St = Stomach; Kid = Kidney; Spl = Spleen; G = Gill; Liv = Liver.

(a)



(b)



(c)

Figure 2.6: Sequence aligment for: **(a)** ATP4A, **(b)** PGC and **(c)** PGA. Generated with Geneious (9.1.5).

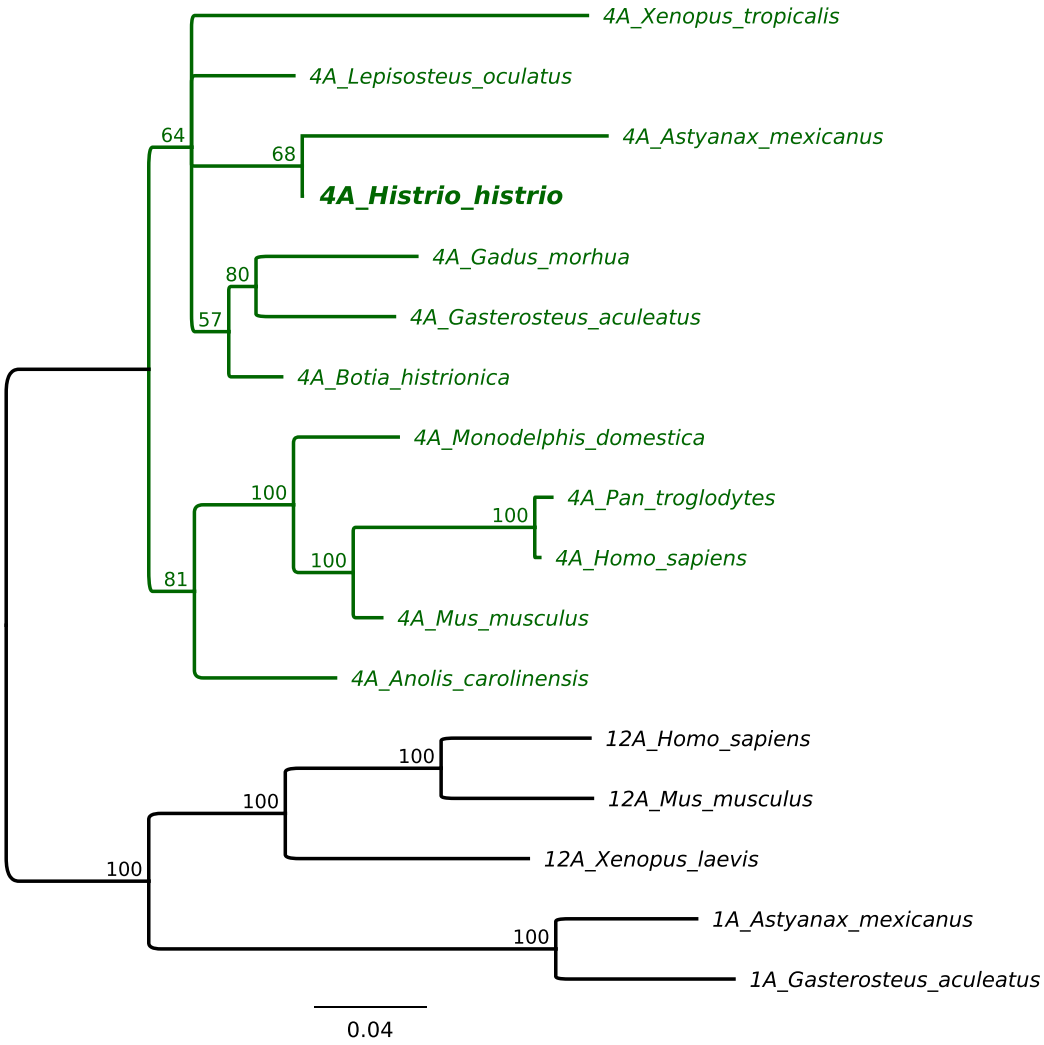


Figure 2.7: Neighbor-Joining tree of *atp4a* gene family. Values at nodes are bootstrap values (100). Generated with Geneious (9.1.5).

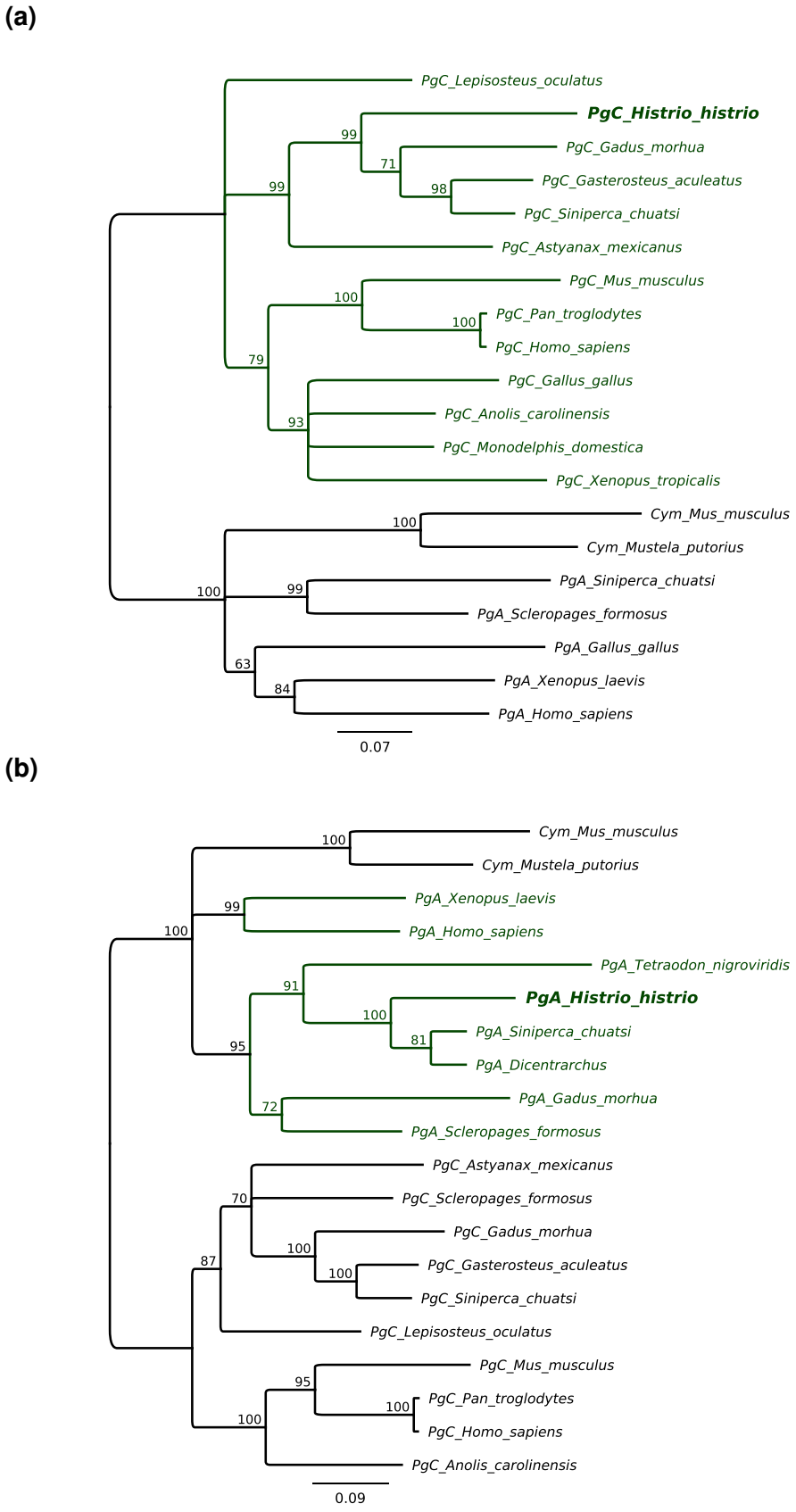


Figure 2.8: Neighbor-Joining tree of **(a)** *pgc* and **(b)** *pga* genes. Values at nodes are bootstrap values (100). Generated with Geneious (9.1.5).

from the analysed sequences are ilustrated in figure 2.6. The phylogenetic analysis of *H. histrio* sequenced genes inferred a grouping with the same genes for other teleost species, as shown in figure 2.7 and figure 2.8. The grouping was found to be highly robust as determined by the bootstrap value.

2.6 Discussion and final remarks

Secondary loss events appeared multiple times since stomach's first emergence with the early gnathostomes (Castro et al., 2014; Wilson and Castro, 2010). Amongst the teleosts there is a specific case that suggest the (post-loss of stomach) replacement of stomach's activity, i.e. acid-pepsin secretion in gastric glands, for a defensive function, it is the case of some Tetraodontiformes (e.g. *Takifugu rubripes*) that use the stomach as a storage place for water that allows for body inflation (Brainerd, 1994; Castro et al., 2014). Despite the absence of the gastric glands (Brainerd, 1994) and the proton pump (Castro et al., 2014), *Takifugu*'s pepsinogens have been identified, however with no digestive function (Kurokawa et al., 2005).

The H^+/K^+-ATPase enzyme is considered the most important component of the system that mediates acid secretion (Podolsky et al., 2015). Nevertheless, this acid secretion implicates high energy cost and a need for a mucous secretion to protect the linning of the stomach and a base secretion neutralise the gastric acid in the intestine. These factors support the advantages of stomach loss in terms of reducing the maintenance costs derived from the digestive process. On the other hand the simple presence of the glands seems to be an advantage for a more efficient digestion, since low-pH not only helps in the denaturation of long proteins but also helps the action of endopeptidases (Castro et al., 2014; Kageyama, 2002; Koelz, 1992).

It is interesting that in the course of evolution, the advantage of a good defence (inflation ability) seems to be valued above a more effective digestion, at least in the case of Tetraodontidae (puffer-fishes) and Diodontidae (porcupine fishes) families.

In this work gastric glands were identified in a still poor studied species, the sargassum fish *Histrio histrio*. The first confirmation of the presence of this glands appeared after an Alcian-blue PAS staining of stomach sections. The PAS staining showed also the presence of basic mucins covering the lumen surface of the stomach, basic mucins are known for a having protective role, i.e. they prevent damage caused by the acid, neutralising it. The

presence of zymogen (pepsinogen that would be later on converted to pepsin) in gastric glands was detected by eosin fluorescence and pointed to glandular activity.

The immunostaining results correlated with the PAS staining of the glands and basic mucins, showing that not only *Histrio histrio* presents a glandular stomach but also that the glands are associated with pumps, figure 2.4.

With the gene and protein expression studies, the full evidence of a functional gastric proton pump, with the secretion of both HCl and pepsinogen, present in *H. histrio*'s stomach was clear. An antibody reactivity was shown in western-blot kidney sample, pointing to a possibility of reabsortion of H^+/K^+ in the kidney tubules as it has been shown for other species (Silver and Soleimani, 1999); the expression of H^+/K^+-ATPase in gills was expected due to its importance at ion and acid/base regulation level (Evans et al., 2005).

In conclusion, the findings reported in *Histrio histrio* counter the hypothesis that, in parallel with the case of *T. rubripes* the acquisition of the inflation ability arose in association to a non-functional stomach. It is expected that, during the course of evolution in these linneages, the ancestors of *T. rubripes* and *H. histrio* have encountered different environmental pressures. If, on the one hand, the *T. rubripes* inhabits not only marine environments but also brackish waters (or even fresh water; Kato et al., 2005; Masuda et al., 1984), on the other hand *H. histrio* is found exclusively in marine water (Gordon, 1938; Pietsch and Grobecker, 1987). They also differ in dietary aspects, *Takifugu* eats mainly algae, mollusks and some crustaceans, while *Histrio* feeding habits include mostly other fishes. A diet rich in calcium carbonate, algae and the presence in less-chloride rich waters[1] are potential drivers for stomach loss (Wilson and Castro, 2010) that are present in *Takifugu rubripes* and not in *Histrio histrio* ancestors. This seems a plausible explanation for the presence of both conditions: gastric and inflation in *Histrio histrio* and for the absence of the gastric phenotype in *Takifugu rubripes*.

The results indicate, in this way, that the inflation ability may not be strictly correlated with a functional loss of the stomach; not being imperatively a driver for stomach loss, neither a result of the acquisition of a non-functional stomach.

[1] brackish and fresh waters have an higher ratio of bicarbonate in their chemical composition when compared with saltwater, while saltwater has an higher ratio of Cl^- ions, (Pilson, 2012)

Chapter 3

Diversity and function of Pleuronectiformes Gastrointestinal tract

3.1 Abstract

Pleuronectiformes are a well studied group and highly exploited in aquaculture and fisheries, giving them a great economic value. It has been reported for this group a gradient in the stomach size across different species. This work aimed to relate this diversity in stomach length with differences in the acidification potential in three species: *Scophthalmus maximus, Solea senegalensis* and *Platichthys flesus*. To this end, *in vitro* and *in situ* acidification experiments were conducted, followed by histological analysis of the gastric glands (through Alcian-blue PAS staining) and immuno detection of the gastric pump. The gastric pump was confirmed for the three species and it was possible to find differences between the analysed species in the *in situ* experiment. This results related the gradient in stomach size with differences in the acidification rates for the studied species.

3.2 Introduction

The gastrointestinal tract (GIT) presents a similar organisation in vertebrates. Nevertheless, some portions of this system, such as the stomach, can differ between groups, being even

absent in some of them (Horn, 1997; Wilson and Castro, 2010). The stomach, and its associated gastric glands and pumps, cannot be limited as an enlargement of gastrointestinal tract (GIT) that serves the function of food storage. It has an important function in the defence from pathogens (Koelz, 1992), enzymatic activity (Barrington, 1957; Koelz, 1992) and can even have some adaptations to air-breathing (e.g. some Siluriformes; Podkowa and Goniakowska-Witalińska, 2003). In fishes, the oxynticopeptic cells secrete both HCl (through the action of H^+/K^+-ATPase) and pepsinogen (a zymogen form of pepsin, a peptidase; Evans et al., 2005; Wilson and Castro, 2010). In order to protect the stomach epithelium from the action of peptidases and acid, a mucous layer (composed by basic mucins) is secreted by the neck cells (inside the gastric pits) and foveolar cells, in the lining of the stomach (Yamazaki et al., 1991).

3.2.1 Pleuronectiformes

The Pleuronectiformes order (flatfishes) is an example of the wide variety in stomach forms. This diversity motivated de Groot (1971) in his work about the diversity of the gastrointestinal tract in the flatfishes. In his work de Groot (1971) described the variation in stomach size and complexity across the Pleuronectiformes order with feeding habits. An extreme case of this diversity seems to be present in the Rhombosoleidae family, where *Ammotretis rostrata* and *Rhombosolea tapirina* were described as agastric by Grove and Campbell (1979).

The flatfishes group include high commercial-valued species used for human consumption, both cultivated in aquaculture or associated to fisheries. In this work three species are focused: two widely known from aquaculture uses, the turbot *Scophtalmus maximus* and the senegalese sole *Solea senegalensis* and one wild species, the flounder *Platichthys flesus*, highly exploited in fisheries activities.

3.2.1.1 *Scophtalmus maximus*

The turbot *Scophtalmus maximus* is a member of Scophthalmidae family with high economic value and widely cultivated in aquaculture facilities across the Iberian Peninsula. In the wild it is found in sandy bottoms of brackish-water habitats, where it feeds mainly on other bottom-living fishes, although it can usually feed on some molluscs and bivalves (de Groot, 1971; Golani et al., 2011; Nissling et al., 2007). In his work, de Groot (1971) included *S. maximus* in the Psettodidae family, and described that this group's gastrointestinal tract (GIT) has a

large esophagus and stomach. When comparing relative lengths of the different components of the *Scophtalmus* GIT, the author found that approximately 30% of it corresponded to the esophagus and stomach (intestine $\approx$ 34.9%).

3.2.1.2 *Platichthys flesus*

Member of the Pleuronectidae family, the flounder *Platichtys flesus* is found in mudy sand bottoms of estuaries and sea. It is higly exploited in fisheries. *P. flesus* are mainly crustacean feeders (de Groot, 1971; Munroe, 2010; Nissling et al., 2007) and were included by de Groot (1971) in a type II group within the Pleuronectidae. This group is characterised for having a smaller esophagus and stomach than Psettodidae and the Pleuronectidae type I (that includes members of the Citharidae family, such as *Brachypleura novaezeelandiae*). The relative length analysis made by de Groot estimated that the esophagus and stomach represented 14.4% of this species GIT while intestine had a value of approximately 58%.

3.2.1.3 *Solea senegalensis*

The senegalese sole, *Solea senegalensis* is a member of Soleidae family, greatly used in aquacultures in Portugal and Spain. In the wild it is an euryhaline polychaete-mollusc feeder (Branco et al., 2010; de Groot, 1971; Monroe et al., 2015). This species was characterised by de Groot (1971) has having a very small esophagus and stomach. The estimated length values were of 10.6% for both esophagus and stomach and approximately 72% for intestine.

Although (de Groot, 1971) provided a complete and well-structured view concerning the correlation between the morphology of Pleuronectiformes' gastrointestinal tract and their feeding habits, it is not yet understood how and at what level, this differences in the stomach size can influence the acid secretion, characteristic from stomach.

3.3 Objectives

This study aimed to understand if the variation in stomach size of three different Pleuronectiforme species, *Scophthalmus maximus, Solea senegalensis* and *Platichthys flesus* affects their stomach acidification rates and reflects differences in their gastric glands.

3.4 Material and methods

3.4.1 Animals

Specimens from each experimental species were acquired: *Scophthalmus maximus* were obtained from an aquaculture facility, Stolt SeaFarm S.A. (Mira, Portugal), *Solea senegalensis* were acquired from an aquaculture stock in CIIMAR and the *Platichthys flesus* were caught in Douro and Minho river. Fishes were kept in re-circulatory systems, at 13℃, under a controlled photoperiod (12^L /12^D hours). Salinity was gradually adjusted to 31 ppt to all species.

Prior to the experiments, animals were fasted for 36 hours in order to normalise the stomach condition, by allowing emptying of any stomach contents.

3.4.2 Stomach acidity experiments: *in situ* and *in vitro*

In order to avoid possible interference of anaesthetic chemicals with the stomach pH, animals were killed by cervical transection (Topic Popovic et al., 2012).

The animals were opened with a ventral incision and the gastrointestinal tract was excised from the terminal part of the esophagus until the anterior part of the intestine.

In situ pH was acquired for a total of 15 animals of each species by placing the pH probe (*Biotrode* Hamilton) against the lumen of the stomach and directly record the pH value from a Radiometer ION85 pH meter, based in Yúfera et al. (2012). Calibration of the probe was made prior to data extraction with pH 4 and 7 standards. Following this procedure, 5 stomachs of each species were stored in fixative for histological analyses while the remaining 10 were used for *in vitro* measurements.

For the *in vitro* assay, a stomach-sac procedure was followed, based in Grosell et al. (2009); Márquez and Fuentes (2014): Stomachs were cleaned by rinsing with mucosal saline, closed at the pyloric sphincter and a cannulae was inserted from the esophagus until the stomach. Stomachs were then filled with mucosal saline and immersed in an aerated serosal saline with carbachol (100 μM), a stimulant of acid secretion (Dharmsathaphorn and Pandol, 1986). The whole system was maintained at 14℃ in order to simulate the fishes' body temperature. The composition of both mucosal and serosal salines is presented in Table 3.1 (adapted from Grosell et al., 2009).

Table 3.1: Mucosal and Serosal Solutions

Salt	Mucosal (g/L)	Serosal (g/L)
NaCl	8.8244	4.5466
MgSO4.7H_2O	0.2218	17.2536
MgCl2.6H_2O	—	6.0990
KCl	0.2237	0.1491
$CaCl_2$	0.111	0.2220
$NaHCO_3$	0.4201	0.0840
Na_2HPO_4	0.0710	—
KH_2PO_4	0.0681	—
HEPES (free acid)	1.3107	—
HEPES (Na salt)	1.4316	—
Urea	0.2703	—
Glucose	0.9008	—

After 2 hours of incubation the mucosal solution was sampled to 2 mL tubes, the pH recorded and then titrated to pH 4 with 0.1 N HCl. The control titrations were made by titrating fresh mucosal saline to the same pH, and at the end, surface area of the stomach was measure.

Net acid flux (nafl) was calculated following the formula presented in equation 3.1 (adapted from Harris, 2007; McDonald and Wood, 1981), and it equals the product of the mucosal incubated inside the stomach - mi (μL) and uEqH (see equation 3.2) divided by the reason between surface contact area (cm^2) and the time of incubation (hours). Negative values in net acid flux represent a net loss of acid by the animal/organ, while positive values represent a net gain of acid.

$$\textbf{nafl}=\frac{\textbf{uEq } \mathbf{H^+} \textbf{ x mi}}{\frac{\textbf{cm}^2}{\textbf{h}}} \tag{3.1}$$

$$\textbf{uEqH}^+\textbf{= (0.1 x tvc - 0.1 x tvic)} \tag{3.2}$$

Equation 3.2: Microequivalent of hydrogen ion per litre **uEq H^+**: **0.1** - concentration of the titrant (M); **tvc** - volume of the titrant used in control saline titration (μL); **tvic** - volume of the titrant used in incubated saline titration (μL).

3.4.3 Histology and Immunohistochemistry

Following fixation in 4% paraformaldehyde/phosphate buffered saline, pH 7.4, for 24 hours at 4℃, tissues were dehydrated through a series of ascending ethanol concentrations, cleared in xylene, embedded in paraffin and sectioned (5 μm thickness) with a Reichert Biocut 2030 microtome. Sections were then stained with Alcian-blue PAS and hematoxylin and images acquired with a Leica DFC300FX digital colour camera mounted on a Leica DM 6000 B microscope.

3.4.3.1 Immunolabelling of gastric pumps

In order to determine the presence of the gastric pumps, an immunolabelling against H^+/K^+-ATPase and Na^+:K^+:Cl^- co-transporter was performed. Paraffin sections (5 μm) were collected onto APS (3-amino propyltriethoxysilane; Sigma-Aldrich, St Louis, MO) coated slides and allowed to air dry. After drying, samples were dewaxed through xylene and ethanol and the sections were circled with a hydrophobic barrier (PAP pen, Sigma-Aldrich). Samples were then blocked with 1% BSA/TPBS (0.05% Tween-20 in Phosphate Buffered Saline (137 mM NaCl, 2.7 mM KCl, 7.8 mM Na_2HPO_4), pH 7.4) for 20 minutes at room temperature. Sections were incubated with a combination of the antibodies T4 (mouse monoclonal) that detects the Na^+:K^+:Cl^- co-transporter (Ip et al., 2013) and C2 (rabbit polyclonal) that detects the H^+/K^+-ATPase (Smolka and Swiger, 1992), diluted respectively at 1:100 and 1:200 in 1% BSA/TPBS, overnight at 4℃ in a humidified chamber. The negative control sections were incubated only in BSA/TPBS. After the incubation, slides were washed with TPBS for 5, 10 and 15 minutes and incubated with goat anti-mouse Alexa Fluor 594 and goat anti-rabbit Alexa Fluor 488 conjugated secondary antibody, both diluted 1:500 (Molecular probes Inc, Eugene, OR) in BSA/TPBS for 1 hour at 37℃. After secondary-antibody incubation, slides were washed again with TPBS. Coverslips were mounted using 1:1 PBS glycerol. The sections were observed on a Leica DM6000 B wide field epi-fluorescence microscope and the images were captured using a cooled digital camera (Leica DFC 340 FX).

3.4.4 Statistics

Statistical distribution of the samples was accessed by a Shapiro test of normality. In order to determine differences between the median of the samples (species surface pH) a non-

parametric Kruskal test was performed followed by a Dunn test. For the *in vitro* analysis an One-way ANOVA followed by a post-hoc Tukey test was performed in order to determine the differences between samples. All statistical analysis were performed using R software, version 3.3.1 (R Core Team, 2016).

3.5 Results

The animals biometrics data was acquired and results are shown in table 3.2, results are presented as means $\pm$ SEM (Standard Error of the Mean).

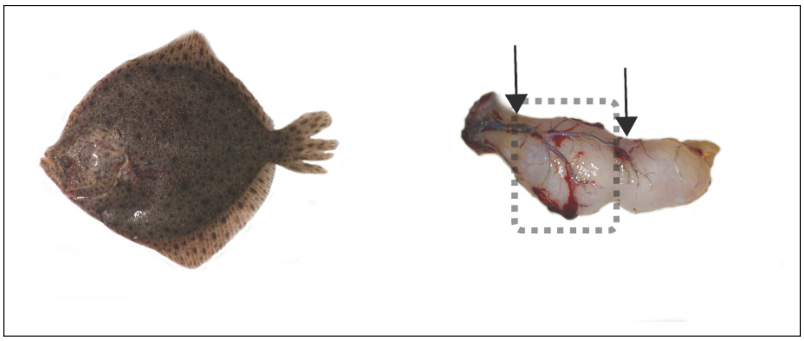
Through a visual analysis of the excised stomachs, it is possible to distinguish a gradient in the gross morphology of this organ through the three studied species. The stomach of *S. maximus* is a robust and well regionalised organ, and it is possible to clearly distinguish, by analysis of external morphology, both its beginning and the end. In the case of *P. flesus*, the stomach is less muscular than in *S. maximus*, but it still has a region delimited by the intersection with the esophagus and with the intestine. In the last studied species, *S. senegalensis*, the stomach appears less visible, and is the shortest among the three species. It is also not possible to distinguish, through external analysis, the esophageal connection, although a clear transition to the intestine is visible (figure 3.1).

Table 3.2: Biometrics of studied species (means$\pm$SEM)

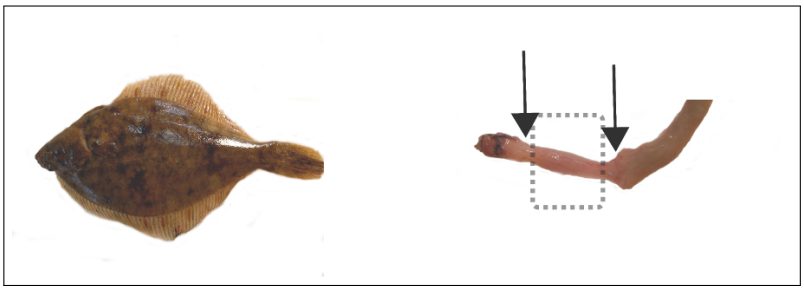
	S. maximus	***P. flesus***	***S. senegalensis***
Height (cm)	46.29 $\pm$ 1.36	24.13 $\pm$ 1.36	26.04 $\pm$ 1.12
Body mass (g)	621.67 $\pm$ 10.52	54.80 $\pm$ 6.11	152.87 $\pm$ 8.29
Stomach mass	3.45 $\pm$ 0.10	3.26 $\pm$ 0.06	2.80 $\pm$ 0.09
Stomach:body mass ratio (%)	0.55 $\pm$ 0.02	6.14 $\pm$ 0.71	1.86 $\pm$ 0.10

3.5.1 Stomach acidification

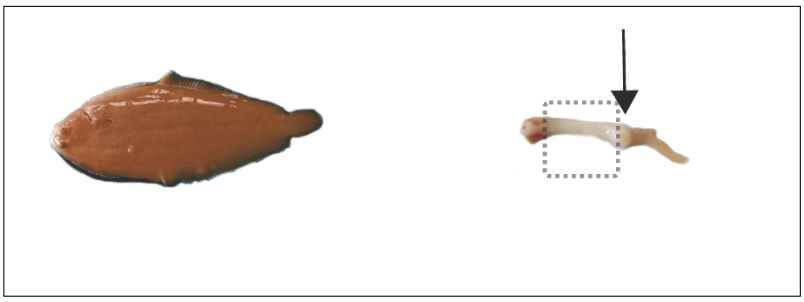
The pH recorded directly at the lumen of the fundic region of the stomach shows statistically significant differences in values across species ($P < 0.05$), being lowest in *Scophtalmus maximus* and highest in *Solea senegalensis*: ***S.maximus*: 6.140 $\pm$ 0.029**; ***P. flesus*: 7.004**



(a) *Scophtalmus maximus*



(b) *Platichthys flesus*



(c) *Solea senegalensis*

Figure 3.1: Comparative view of the species studied and their stomachs, images are not to scale. Dashed square indicates the stomach and arrows the esophageal and pyloric regions (left and right, respectively). The *Solea senegalensis*' stomach presents a blurry esophageal transition.

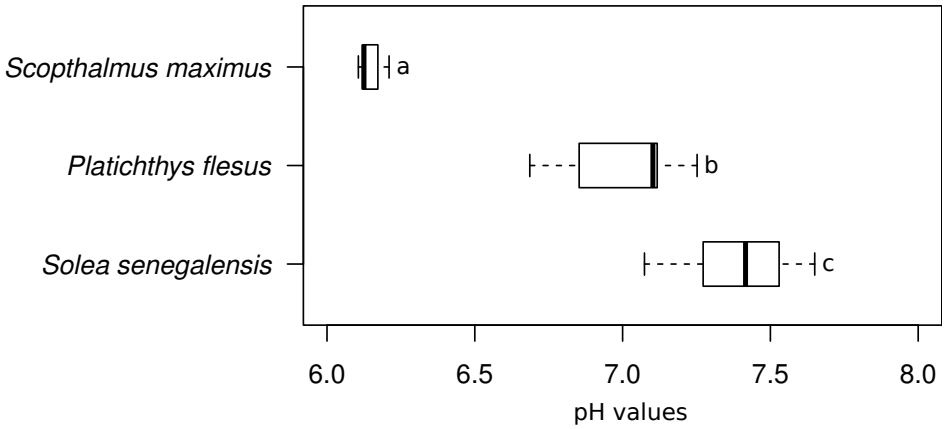


Figure 3.2: Boxplot of the distribution of pH values recorded in the *in situ* experiment. Bars with different letters are significantly different from each other. n= 15, data distribution: *Scophtalmus maximus*: minimum= 6.106, Q1= 6.113, Q2 (median)= 6.125, Q3= 6.172, maximum= 6.21; *Platichthys flesus*: minimum= 6.686, Q1= 6.787, Q2 (median)= 7.103, Q3= 7.180, maximum= 7.252; *Solea senegalensis*: minimum= 7.074, Q1= 7.21, Q2 (median)= 7.416, Q3= 7.545, maximum= 7.65.

Table 3.3: Net acid flux results

	nafl (uEqH^{+}.cm^{-2}.h^{-1})
S. maximus	-0.358 $\pm$ 0.16
P. flesus	-0.416 $\pm$ 0.15
S. senegalensis	-0.174 $\pm$ 0.28

$\pm$ **0.180**; ***S. senegalensis*****: 7.400** $\pm$ **0.143**. This distribution of pH values is illustrated in figure 3.2.

In the *in vitro* experiment, after 2 hours of incubation, the pH of the mucosal saline was measured and the results show a significant higher pH in *S.maximus* and *P. flesus* ($P < 0.05$) than the pH registered for control saline: ***S. maximus*****: 7.267** $\pm$ **0.022**; ***P. flesus*****: 7.293** $\pm$ **0.024**; ***S. senegalensis***: 7.063 $\pm$ 0.043; **Control**: 7.090 $\pm$ 0.010. Incubated and control salines where then titrated to a pH of 4. The values obtained for the net acid flux are present in table 3.3, results are presented as means $\pm$ SEM.

The negative values indicate that acid (HCl) was uptake or base was excreted. No significant differences were found between species ($P < 0.05$). Distribution of the results are illustrated in figure 3.3.

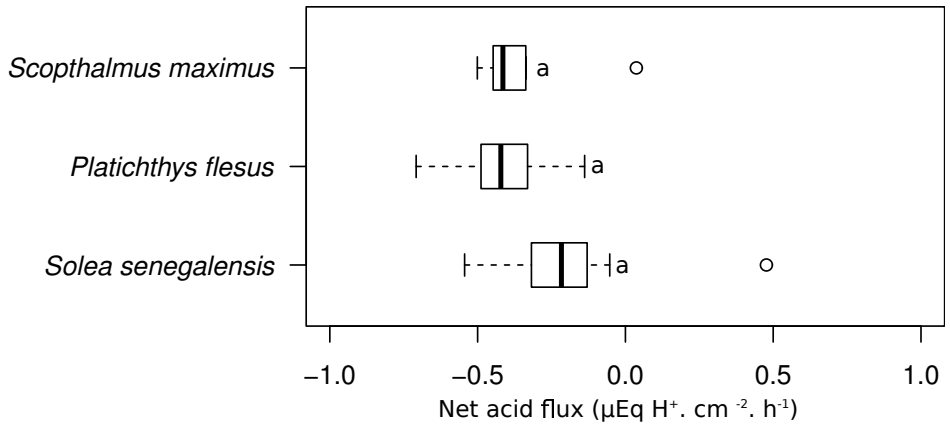


Figure 3.3: Boxplot of the net acid flux values obtained for the different species, *in vitro* experiment. Bars with same letters are not significantly different from each other. n= 10, data distribution: *Scophtalmus maximus*: minimum= -0.501, Q1= -0.452, Q2 (median)= -0.414, Q3= -0.336, maximum (excluding outliers)= -0.336; *Platichthys flesus*: minimum= -0.708, Q1= -0.518, Q2 (median)= -0.422, Q3= -0.315, maximum= -0.138; *Solea senegalensis*: minimum= -0.544, Q1= -0.407, Q2 (median)= -0.2165, Q3= -0.053, maximum (excluding outliers): -0.053.

3.5.2 Histology

Through the analysis of Alcian-blue PAS stained sections of the stomachs from the different species studied, it is possible to identify the gastric glands and a basic-mucin layer in the epithelium lining the stomach. This mucopolyssaccharide layer appears less demarcated in *Solea senegalensis* sections (figure 3.4 a,c,e). The immunolabeling of H^+/K^+-ATPase is visible for all species, showing the presence of the gastric pump in the gastric glands. The Na^+:K^+:Cl^- co-transporter appears in a basolateral position in gastric gland cells (figure 3.4 b,d,f).

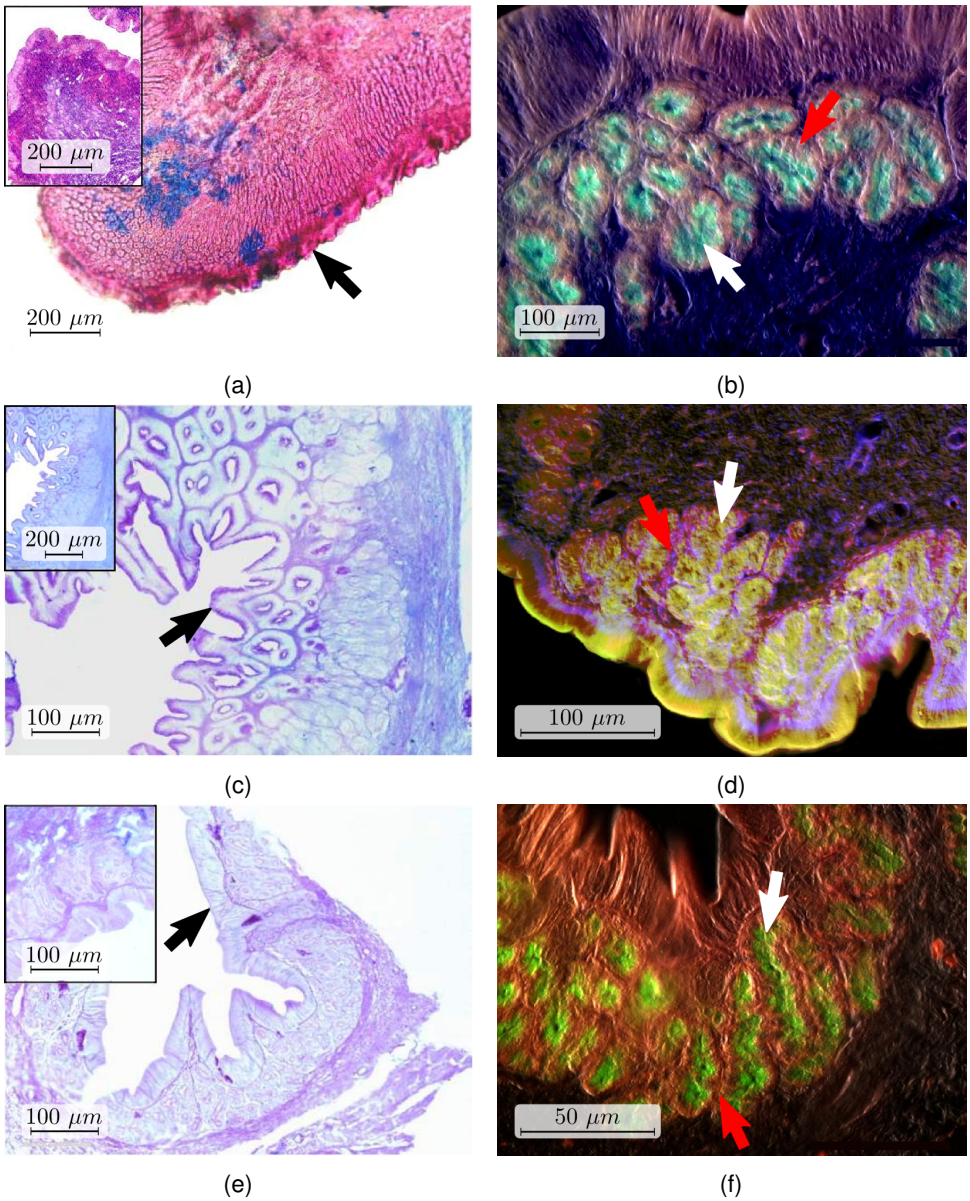


Figure 3.4: On the left: Alcian-blue PAS staining of stomach sections from **(a)** *Scophtalmus maximus*; **(c)** *Platichthys flesus* and **(e)** *Solea senegalensis*, showing a epithelial mucin layer (black arrows). On the right: Imuno-labeling of the Gastric proton pump H^+/K^+-ATPase (colored green, white arrows) and of the Na^+:K^+:Cl^- co-transporter (colored red, red arrows) in **(b)** *Scophtalmus maximus*; **(d)** *Platichthys flesus* and **(f)** *Solea senegalensis*.

3.6 Discussion and final remarks

Since its first appearance, more than 400 million years ago, the stomach suffered multiple evolutionary pressures that led to a significant diversity of forms. Multiple events of gene loss are associated with the absence of the stomach phenotype in several vertebrate groups, most of them fishes. In the specific case of the Pleuronectiformes order, stomach loss is believed to have occurred in the Rhombosoleidae family. Additionally, a noticeable gradient in stomach size across all Pleuronectiformes order was demonstrated by de Groot (1971). In this way, this work aimed to test the hypothesis that the reported difference in size, (de Groot, 1971), is accompanied by differences in the acidification rates of the stomach, as a direct measurement of proton pump (H^+/K^+-ATPase) activity. To this end, *in vitro* and *in situ* acidification experiments were conducted. Furthermore, histological visualisation of gastric glands and basic mucins was obtained by Alcian-blue PAS staining and in order to access the presence of the gastric pump in an immunohistochemical localisation of the H^+/K^+-ATPase was performed.

The *in situ* experiment showed an higher pH of stomach surface for *S. senegalensis*, values are in accordance to the findings of Yúfera and Darias (2007); the lowest pH was recorded to *S. maximus* relating to the bigger and musculated stomach of this species. Although the *in vitro* experiment didn't result in the acquisition of data that directly connects to differences in luminal acidification capacity in the three species, the results obtained point to an important role of basic mucin secretion.The initial pH of the saline, after the 2 hour incubation, in *S. maximus* and *P. flesus* was higher than the pH from control saline, and thus an alkalinization of the solution due to mucin secretion in the lumen during the incubation is probable to have occurred. No differences in acid excretion rates were found in the analysis of the titrated salines, however negative values correlates with base excretion during the incubation period.

Through a histological analysis, the mucin layer in the lining of *S. senegalensis* appears less demarcated in comparison with the other studied species, correlating with the pH experiments results. The gastric proton pump was identified in *S. senegalensis* demonstrating a fully-functional stomach for this species although its apparent lack of acidification.

Multiple pressures are believed to be capable of modulate the evolution and diversification of the stomach phenotype. The diet has been pointed as one of this possible factors in the case of organisms that feed on mollusks and other preys with high bicarbonate content (as crustaceans). Moreover, diets based on low nutritional material (as in the case of bottom

feeders) and herbivory are also susceptible of modulate the stomach phenotype (Lobel, 1981; Wilson and Castro, 2010). Additionally, it has been suggested that carnivores and omnivores would have lower luminal pH since microorganisms present in their preys are presumably more harmful to the predator than microorganisms present in plants (Davies and Pedersen, 2008).

These environmental pressures are likely to be involved in the diversity in stomach size of the species studied in this work. *S. maximus*, the species presenting the apparently stronger stomach (both in terms of morphology and function, i.e. acidification) is mainly a fish feeder (de Groot, 1971), while the two other studied species, *P. flesus* and *S. senegalensis* are essentially crustacean and polychaete feeders, respectively (Branco et al., 2010; de Groot, 1971; Monroe et al., 2015; Munroe, 2010; Nissling et al., 2007).

It is interesting to compare the digestion in *S. senegalensis*, that mainly occurs in the proximal section on the intestine (Yúfera and Darias, 2007) with the case of the agastric Cypriniformes where the digestion also occurs in this portion of the GIT (German, 2009). This leaves as an open question the possibility of this species be in a modulation process for the agastric phenotype. In this work the tested hypothesis of the stomach size gradient across the three species studied would be related to differences in acidification and in their gastric glands was partially proven through the *in situ* and histology analysis, although no significant results pointed to differences in the *in vitro* experiment. The lower pH values recorded in the analysed *S. maximus* correlate with the previous studies that related the carnivorous diet with a higher luminal acidification (Davies and Pedersen, 2008; de Groot, 1971).

A broader analysis will be of great importance to deepen our understanding of implications on stomach diversity implication across Pleuronectiformes, namely integrating a protein quantitative analysis, Western-blot, phylogenetic analysis and a higher number of flatfish species.

Chapter 4

Final Considerations and Future Perspectives

The stomach origin can be traced back to more than 400 million years ago (Brazeau and Friedman, 2015) to the emergence of the early gnathostomes. Across most of the vertebrates, the gastrointestinal tract comprises the same basic components: esophagus, stomach, intestine and rectum (Stevens and Hume, 2004). Nevertheless, the stomach presents a remarkable phenotypic diversity in vertebrates (Smith et al., 2000; Wilson and Castro, 2010). The stomach's primary functions are the storage, mixing and primary digestion of food (Horn, 1997); however, it is the later acid-peptic digestion that defines the vertebrate stomach (Koelz, 1992). The first phases of digestion that occur in this organ and are associated to an acid secretion that stimulates the peptic breakage of food. The gastric acid secretion and its interactions with peptidases (i.e. pepsinogens) were first described in the 18^{th} century, and helped guide research for the following two centuries (Sachs et al., 1995).

Great attention has been paid to the mechanisms of evolution by gene duplication and its role in the emergence of new animal features (Blomme et al., 2006; Dehal and Boore, 2005). Despite efforts to elucidate the impacts of gene duplication, the loss of genes and its role in phenotype evolution have received far less attention (Albalat and Cañestro, 2016). Gene loss can appear either as a result of an abrupt mutation (e.g. failure in meiosis crossing-over mechanisms or viral delection of the gene from the genome), or as the product of a long process of pseudogenization (by the consecutive accumulation of mutations in the genomic code). In this last case, nonsense mutations, insertions and/or deletions alter the transcripts. Thus, the gene expression is completely changed, leading to the silencing of one or several

metabolic mechanisms and organism functions (Albalat and Cañestro, 2016). There is compelling evidence that multiple events of stomach loss happened in several vertebrate lineages and, particularly, in teleosteans (Castro et al., 2014; Day et al., 2011; Ordoñez et al., 2008). Accompanying loss events, new functions seem to have appeared. As is the case of the Tetraodontidae family (Tetraodontiformes order) in which the gastric function was replaced by a defensive one, as the stomach, without the presence of any gastric glands or proton pumps, allows for the inflation behaviour by a sudden water intake (Brainerd, 1994; Wainwright and Turingan, 1997; Wilson and Castro, 2010). In the case of some catfishes, from Loricariidae and Trichomycteridae families, the stomach presents reduced gastric glands and has acquired an additional respiration function (Wilson and Castro, 2010).

Although a functional stomach presents unarguable advantages in improving peptic digestion, facilitating the releasing of free amino acids, improving PO_4^- and Ca^{2+} uptake, and in protecting the organism from external hazards (e.g microorganisms; Castro et al., 2014; Horn, 1997; Kageyama, 2002; Koelz, 1992), selective pressures may have lead to stomach loss events. Several hypotheses for drivers of stomach modification and loss have been suggested, including alimentary habits (diets rich in bicarbonate could neutralise the luminal pH; Lobel, 1981), the high energy cost of stomach digestive process and the replacement by other more cost-effective functions. Studies indicate that the stomach loss phenotype is correlated with the absence of gastric proton pump genotype (*ATP4A* and *ATP4B* genes) and pepsinogen expression (Castro et al., 2014). The gastric proton pump (H^+/K^+-ATPase) and pepsinogen represent good estimators of a functional stomach in complementing histology techniques that allow for the identification of gastric glands and protective mucins.

This work had as its primary and broader objective the exploration of the diversity of the stomach in teleosteans, using two case studies. The first case, concerning the sargassum fish *Histrio histrio*'s stomach histological and molecular analysis, revealed a fully functional stomach with a defensive function - inflation. Thus, unlike the pufferfishes, which have lost their gastric glands and *atp4a* genes (Brainerd, 1994; Castro et al., 2014), the sargassum fish has not.

The second case study aimed to explore how the morphological diversity of stomachs in the Pleuronectiformes order reflects differences in luminal acidification and in gastric gland development. It was possible to negatively correlate differences in the surface pH of the stomach and positively correlate mucin production to the different gradients of stomach size. However, further studies are needed to reinforce these relationships.

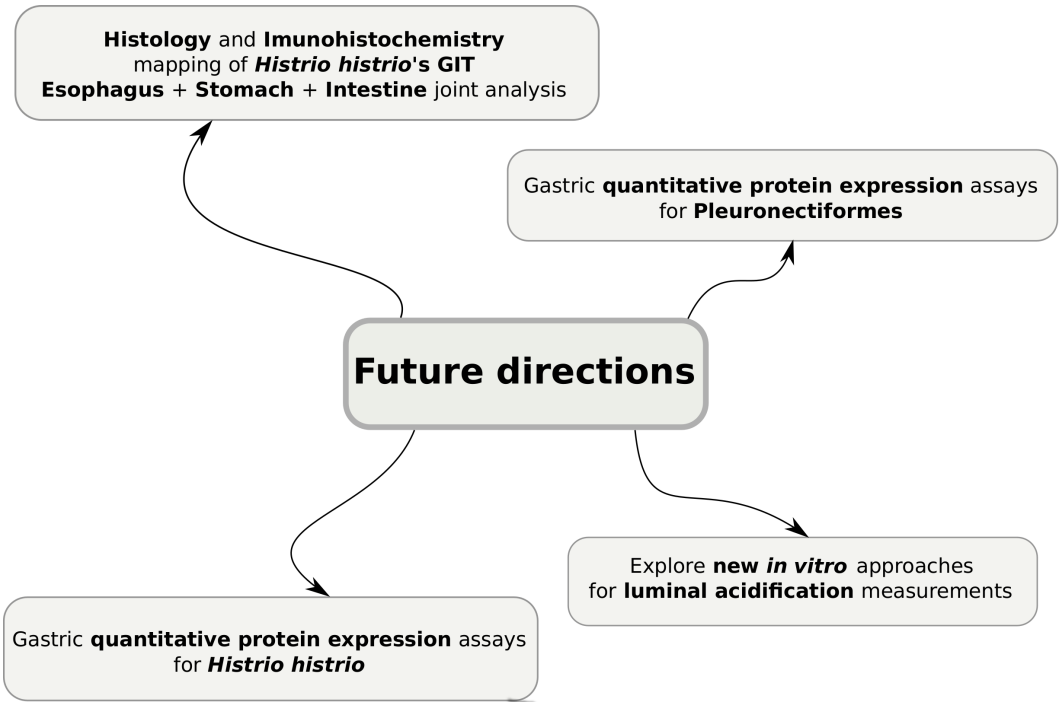


Figure 4.1: Future directions proposed to this work.

Some study limitations can be pointed out in this final reflection, specifically relating to the study of stomach diversity amongst Pleuronectiformes. Ideally, the number of species of flatfishes studied should be enlarged in order to attempt to have more representative species of each diet. Moreover, all animals should have been collected in the wild in order to prevent possible effects of modulation by successive generations under cultivated aquaculture conditions. Obviously, several economic and time consuming aspects were determinant in the experimental design of the present experiments. Nevertheless this work provided good insight into the explored aspects and opened a window to future projects where limitations can be fixed. Several future approaches have been delineated, such as the development of new techniques to quantify luminal acidification and the gastric protein quantification through Western-blot (figure 4.1).

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