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First insights about orexigenic activity and gastrointestinal tissue localization of ghrelin from Corvina drum (*Cilus gilberti*).

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ABSTRACT

The croaker *Cilus gilberti* commonly known as Corvina drum is considered a target marine species for the diversification of Chilean aquaculture. To optimize culture conditions, molecular markers for appetite regulation must be examined. Ghrelin, a gastrointestinal peptide, plays a stimulatory role in the food intake of mammals and teleost fish. Nevertheless, even though the appetite-controlling system is considered relatively well conserved among vertebrates, the bioactivity of these molecules should be analyzed in each fish species. Therefore, this study aimed to investigate the expression and orexigenic ability of *C. gilberti* ghrelin. After the pre and postprandial period the stomach expression of ghrelin mRNA in juvenile *C. gilberti* was analyzed. The coding sequence of *C. gilberti* ghrelin was used to identify the mature peptide and then chemically synthesized. The orexigenic ability of acylated ghrelin (cgGhre) and non-acylated (D-cgGhre) ghrelin was tested in *C. gilberti* juveniles. Moreover, the blood and the gastrointestinal location of synthetic ghrelin after intraperitoneal injection were measured. The results showed that the stomach has the highest expression of ghrelin mRNA, and that ghrelin levels increased in the preprandial period and diminished after it. There were no differences in the secondary structure of D-cgGhre compared to cgGhre but the peptide with the serine acylation stabilized its unordered conformation. However, the highest cumulative feed intake occurred in fish intraperitoneally injected with cgGhre. In addition, synthetic ghrelin was maintained in the *C. gilberti* blood until 8 h post-injection (hpi). Finally, biotinylated ghrelin allowed localizing the synthetic peptide in digestive tissue, mainly in the stomach and pyloric caeca. The mRNA expression of the growth hormone secretagogue receptor (GHS-R), also known as the ghrelin receptor, in gastrointestinal organs supports the idea of peripheral orexigenic regulation in these tissues. In conclusion, results suggest that *C. gilberti* ghrelin conserves the orexigenic ability reported in other teleost fish with a regulatory role in the gastrointestinal tract (GIT) of the Corvina drum. This is the first report demonstrating the uptake and distribution of a small orexigenic peptide in the digestive tissues of a South American sciaenid. Although ghrelin is a promising molecular marker for feed intake analysis in *C. gilberti* culture, further research is needed to continue evaluating the effects of aquaculture practices on peripheral appetite signalizer.

Keywords: Ghrelin; *C. gilberti*, orexigenic; GHS-R receptor, gastrointestinal tissue.

1. INTRODUCTION

In all organisms, the energy for survival is provided through the intake and absorption of food. Therefore, animal growth depends on its capacity to digest and assimilate the ingested nutrients. In fish aquaculture, feed cost represents the highest share in the total cost during the production period, 30–60% of it (Luna et al., 2019). Therefore, minimizing losses during feed intake plays an important role in the success of the fish aquaculture industry. Moreover, aquaculture is looking for economic sustainability based on increased production efficiency. As a result, knowledge about nutrient requirements, effective feed ingredients, additives, and new practices in feed management is highly relevant (Boyd et al., 2020). Within the practices of feed management, the identification of molecular markers associated with feeding signals in aquaculture species is essential for managing feeding periods, photoperiod, and specie-specific diet evaluation.

In fish, as in mammals, neuronal systems involved in food intake regulation are morphologically and functionally interconnected, forming a network from the appetite brain signals in the hypothalamus to the peripheral signals from gastrointestinal tissues, incorporating the energy status and hunger/satiety signals (Rønnestad et al., 2017). Many molecules of this process, firstly identified in higher vertebrates, have also been described in teleost fish. Orexigenic neuropeptides, such as neuropeptide Y and ß-endorphin, are probably acting in brain areas associated with increasing appetite regulation (Lin et al., 2000; Volkoff, 2016). In addition, gastrointestinal tract (GIT) orexigenic/anorexigenic peptides (GIT-peptides) are involved in the peripheral control of meal processing and GIT-brain signaling associated with decreasing or increasing appetite (Tinoco et al., 2014; Unniappan et al., 2004; Unniappan and Peter, 2005; Volkoff et al., 2005).

In teleost fish, the most evidence of GIT-peptides function is based on the direct injection of peptides in the fish and analysis of the amount of feed eaten. However, different authors describe orexigenic or anorexigenic properties for the same peptide (Jönsson et al., 2010; Tinoco et al., 2014). Therefore, even though the appetite-controlling system is considered to be relatively well conserved among vertebrates, studies have shown that in fish, the corresponding hormones and peptides orthologues may differ in their functions, based on the differential morpho-physiological response of their digestive tract (Volkoff, 2016). Thus, an appropriate description of the activity and function of these molecules in fish should be analyzed for each fish species.

Several reports describe that the GIT- peptide ghrelin plays an orexigenic stimulatory role in food intake in mammals and teleost fish (Tinoco et al., 2014; Unniappan et al., 2004; Unniappan and Peter, 2005; Volkoff et al., 2005). The ghrelin mRNA encodes a preprohormone, including a signal peptide, mature peptide, and C-terminal regions. Thus, post-translational processing results in the formation of biologically active ghrelin. Although the major active product in high vertebrates is the 27-28 amino acid peptide

(called simply ghrelin), the ghrelin propeptide can generate a second peptide named ghrelin-associated peptide, which has already proved to be biologically active in high vertebrates modulating the action of ghrelin (Delporte, 2013).
The biologically active ghrelin in teleost is a peptide with 12-25 amino acid residues depending on the species (Bertucci et al., 2019, Jönsson et al., 2010; Kaiya et al., 2003a). Despite the variable number of amino acids, the ghrelin sequence is relatively well conserved between different fish species, particularly at the amino terminus. These first four amino acids form the active center of the peptide, characterized by the acylation of a third serine residue, mainly n-octanoylated serine (acylGhr) (Kaiya et al., 2003b). Therefore, the functional activity of fish ghrelin has been assessed using its acylated-molecular form because different authors assume that the non-acylated ghrelin form (DesAcylGhre) is inactive, which is still controversial. In fact, research in humans showed the blood circulation of both molecular forms of ghrelin (Holmes et al., 2009).

The biological activity of ghrelin stems from its ability to bind and activate GHS-R receptors, described as growth hormone secretagogue receptors (Kaiya et al., 2010). Two GHS-R have been described in vertebrates: one is a functional G-protein-coupled receptor with seven transmembrane domains, named GHS-R1a, whereas the other is a truncated form of GHS-R1a lacking transmembrane domain 5, named GHS-R1b (Albarrán-Zeckler and Smith, 2013). Different authors have reported these GHS-R isoforms in teleost fish such as black seabream, goldfish, and zebrafish (Kaiya et al., 2013, 2010; Perelló-Amorós et al., 2019; Small et al., 2019). According to gene expression analysis, the GHS-R mRNA is produced in many tissues of teleost fish, including those critical in peripheral and central feeding regulation (e.g., the hypothalamus, liver, and gastrointestinal tract) (Kaiya et al., 2010). Thus, the mRNA expression of GHS-R in gastrointestinal tissue supports the idea that fish ghrelin is a peripheral signalizer of appetite regulation. However, there are no studies at the protein level evaluating the presence of ghrelin peptide on fish gastrointestinal tissues.

Although digestion starts when feed reaches the digestive tract, an efficient digestion requires some pre-ingestion steps and, in this sense, the physiological anticipation to the availability of food depends on internal signals in the gastrointestinal tract (Yúfera et al., 2014, 2012). Therefore, the design of optimal daily feeding protocols promoting more efficient feed digestion requires a more profound knowledge on how the feeding time and frequency may influence the daily different factors of the digestive process, such as the molecular expression of GIT-peptides. An increase of ghrelin transcript in the gastrointestinal and brain tissues after the preprandial and fasting periods and a decrease after food intake has been evidenced in vertebrates (Ariyasu et al., 2001; Cummings, 2006; Fox et al., 2009; Reynolds et al., 2010). However, the results obtained in fish are not as consistent as those observed in mammals (Hevrøy et al., 2011; Jönsson et al., 2007; Pankhurst et al., 2008; Peddu et al., 2009; Unniappan et al., 2004). Therefore, the

expression of this orexigenic peptide should be evaluated in each fish species.

Currently, the croaker *C. gilberti* is considered a target marine species for the diversification of fish aquaculture by the Chilean government (Álvarez et al., 2020). In order to support feeding protocols of this species, it is necessary to establish physiological markers for the development in captivity, which is possible through the knowledge of the main signalizers of physiological response to appetite regulation. Therefore, this study aimed to investigate the expression and orexigenic activity of *C. gilberti* ghrelin. After the pre and postprandial period, the stomach expression of ghrelin mRNA in *C. gilberti* juveniles was analyzed. The coding sequence of ghrelin was used to identify the mature peptide and then it was chemically synthesized, containing or not the serine acylation. Then, the orexigenic ability of two ghrelin molecular forms was tested in *C. gilberti* juveniles. Finally, the gastrointestinal location and plasmatic acylGhr were measured after intraperitoneal injection of *C. gilberti* juveniles.

2. MATERIALS AND METHODS

2.1 Peptide synthesis and characterization.

Synthetic ghrelin peptides were synthesized by solid phase multiple peptide system using the Tea Bag strategy as previously described (Guzmán et al., 2021). The peptide containing acylation was obtained by incorporating in the synthesis Fmoc-Ser(N-Octanoyl)-OH. Moreover, a portion of peptide-resin was used for biotin coupling. Then the peptides were cleaved with TFA/TIS/EDT/H_20 (92.5/2.5/2.5/2.5) (trifluoroacetic acid/triisopropylsilane/1.2-ethanditiol/ultrapure water) and purified by RP-HPLC with a 0-70% acetonitrile-water mixture gradient over 30 min at a flowrate of 1 mL/min. Additionally, the peptides coupled with biotin were applied onto a Sep-pak C18 Vac cartridge (Waters Associates) equilibrated in acidified water (0.05% trifluoroacetic acid in UPW-Ultra Pure Water). After washing with acidified water, the peptides were eluted at a flowrate of 1 mL/min with 10%, 30%, and 100% acetonitrile (ACN). The appropriate fractions were collected and the ACN was evaporated on a SpeedVac centrifugal evaporator. The peptides were lyophilized and analyzed by ESI-MS mass spectrometry to confirm their molecular masses.

The secondary structure of the synthetic peptides was analyzed by circular dichroism (CD) spectroscopy on a JASCO J-815 CD Spectrometer (Jasco Corp., Tokyo, Japan). CD spectra of the peptides were recorded in Milli-Q water and trifluoroethanol (TFE, 30% v/v in water) in the far ultra-violet (UV) range (190-250 nm), using quartz cuvettes of 0.1 cm path length and 1 nm bandwidth at 0.1 nm resolution. The solvent contribution blank was subtracted from each sample spectrum. Molar ellipticity was calculated for each peptide.

2.2 Fish maintenance and bioassays

Cilus gilberti juveniles (n=200; 90-100 g) were obtained from Fundación Chile facilities in Tongoy Bay, Coquimbo, Chile, and transferred to the Fish Farm Laboratory of Universidad Católica del Norte at Coquimbo. Fish were held in six circular fiberglass tanks of 0.5 m^3 each containing 25 fish with oxygen pumps, biological filters, and ultraviolet sterilizing units and flow-through seawater. Fish were fed to satiation (commercial diet of Skretting Supreme RC100 with 45% protein and 20% lipids) thrice daily (09:00; 14:00 and 17:30 h) by hand from the beginning of January until the beginning of February 2021, which was considered as the adaptation period before the experiment. During the experimental period, dissolved oxygen (DO) (7.3 mg/mL ±0.5) and temperature (17.1 °C ±1.2) were tested daily using a multi parameter probe (YSI, Inc.). Fish were held under a 12L:12D photoperiod. No variations in water quality parameters were detected among experimental groups. Neither chemical nor antibiotic treatments were used at any time.

2.2.1 Tissue sampling

After the adaptation period, six *C. gilberti* juveniles were captured (two random fish from three different tanks) and sacrificed by an anesthetic overdose (200 mg/L of Tricaine MS-222 in seawater). Different tissue portions (brain, stomach, liver, intestine, white muscle, pyloric caeca and kidney) for RNA extraction were placed in sterile tubes containing 400 µL RNAlater® (Thermo Fisher) and then stored at − 80 °C prior to analysis.

2.2.2 Pre-postprandial study

After the adaptation period, other group of juveniles was divided into two groups of four tanks: one for preprandial and another for postprandial sampling. This ensured that the fish sampled post-prandially were not disturbed by the preprandial sampling and therefore had normal feeding behavior (Vilasa et al., 2015). Preprandial sampling of 2 fish per tank was conducted at one hour before the start of the first daily meal, whereas, the postprandial sampling of 2 fish per tank was conducted 1 h and 6 h after the first morning meal. Sampled fish were captured and sacrificed by an anesthetic overdose (200 mg/L of Tricaine MS-222 in seawater). Registration of body weight (BW) and length were done when fish were sedated. In addition, blood samples were taken from the caudal vein with heparinized 1 mL syringes and then centrifuged at 5,000 g for 5 min at 4 °C, the plasma fraction being stored at -20 °C. Stomach tissue portions for RNA extraction were placed in sterile tubes containing 400 µL RNAlater® (Thermo Fisher) and then stored at − 80 °C prior to further analysis.

2.2.3 Effect of molecular forms of ghrelin in the *C. gilberti* feed intake.

Another group of *C. gilberti* juveniles were used to intraperitoneal (IP) injection of ghrelin molecular forms. The experiment was performed following the doses and procedures described by Yuan et al. (2015). Briefly, 72 fish were anesthetized with 20 mg/L of Tricaine MS-222 in seawater and were divided into three groups that received IP injection: cgGhre at 100 ng/g BW, D-cgGhre at 100 ng/g BW, and one control group, which received

IP injection of the same volume of teleost saline (sterile 0.65% NaCl). Each group of fish were randomly distributed in four tanks of 100 L each containing 6 fish. One hour after IP injection, each fish was fed to apparent satiation with a commercial diet of Skretting. Uneaten feed was siphoned out of each tank 1 h after each feeding operation according to a daily fed protocol of *C. gilberti*. The unconsumed feed was separated and dried overnight at 50 °C. Food intake is expressed in milligrams of food consumed per gram wet BW.

2.2.4 Location of *C. gilberti* ghrelin in gastrointestinal tissues and blood.

Another group of *C. gilberti* juveniles was used to follow the location of biotinylated ghrelin. 40 fish were anesthetized with 20 mg/L of Tricaine MS-222 in seawater and were divided into two groups that received IP injection: biotinylated cgGhre at 100 ng/g BW, and one control group (same volume of teleost saline). Each group of fish was randomly distributed in two tanks of 100 L each, containing 10 fish. After 2 and 8 hours of IP injection, three fish in each tank were captured and sacrificed by an anesthetic overdose (200 mg/L of Tricaine MS-222 in seawater). Registration of body weight (BW) and length was done when fish were sedated. In addition, blood samples were taken from the caudal vein with heparinized 1 mL syringes and then centrifuged at 5,000 g for 5 min at 4 °C, the plasma fraction being stored at -20 °C. Stomach, pyloric caeca, gut and liver tissue portions for histochemical analysis were placed in paraformaldehyde solution 4% in saline phosphate buffer (PBS) pH 7.4, and stored for further analysis.

2.3 Cloning and bioinformatics analysis of *C. gilberti* ghrelin nucleotide sequence.

The partial coding sequence of *C. gilberti* ghrelin (cgGhre) was obtained by contigs sequences search from *C. gilberti* (Fundacion Chile). Primers designed using the Primer3 Input software for the amplification of these sequences is listed in Supplementary Table 1. Stomach cDNA templates were used to amplify cgGhre. A 333-bp PCR product were then purified using a E.Z.N.A.® Gel Extraction Kit (Omega Biotek). The amplicon sequence was verified by sequencing (Macrogen Inc., Seoul, Korea). Sequence homology was established using BLAST (http://blast.ncbi.nlm.nih.gov/). The putative protein sequence was obtained from the Expasy portal (http://www.expasy.org/). Multiple alignments were performed using the ClustalW tools in MEGA 6.0 software. The estimation of evolutionary divergence between nucleotide sequences matrix for teleost propeptide ghrelin are listed in Supplementary Table 2.

The phylogenetic analysis was performed using Mr.Bayes v3.2.6 online Bayesian Inference of Phylogeny (http://www.phylogeny.fr/one_task.cgi?task_type=mrbayes); this program uses Markov chain Monte Carlo (MCMC) methods to estimate the posterior distribution of model parameters (Ronquist and Huelsenbeck, 2003).

Bioinformatics tertiary structure models of putative mature ghrelin from *C. gilberti* was analyzed in PEP-FOLD3 web server (http://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD3) (Lamiable et al., 2016). After choosing the best model, the

3D structure of peptide was constructed using PyMOL. For the phylogenetic analysis, amino acid sequence homologues in other species (fishes and mammalian) were retrieved form the National Centre for Biotechnology Information (http:/www.ncbi.nlm.nih.gov/) database, using the *C. gilberti* sequence as query.

2.4 Gene expression analysis by RT-qPCR

Specific primers were designed to amplify ghrelin and ghrelin receptor mRNA from *C. gilberti* (Supplementary Table 1). In order to validate the β-actin as a housekeeping gene for the samples, statistical tests on β-actin expression values among different conditions were performed. No-significant differences were found among them ($P > 0.05$). RT-qPCR was performed using 20 µL of reaction mixtures containing Takyon™ SyberGreen/ROX qPCR Master Mix (EUROGENTEC, USA), 0.3 µM (final concentration) of each primer, and 2 µL of cDNA. Primer pair efficiencies (E) were calculated from the given slopes according to the equation: $E = 10^{[-1/slope]}$. Assays were carried out in an Mx3000P qPCR System (Agilent Technologies) with an initial denaturation step of 10 min at 95 °C followed by 40 PCR cycles of denaturation step (95 °C, 15 s) and annealing-extension step (60 °C, 1 min). Finally, the melting curve was obtained at 75-95 °C with a heating rate of 0.1 °C per second and continuous fluorescence measurement. Relative expression was calculated using the -2 ΔΔCq method (Livak and Schmittgen, 2001) using the measured quantification cycle (Cq) values of β-actin housekeeping gene to normalize the measured Cq values of target gen.

2.5 Biotinylated ghrelin quantification in plasma.

Total protein in plasma samples was determined using the bicinchoninic acid assay (Pierce BCA protein assay kit, Thermo Scientific, USA) using bovine serum albumin as ~~the~~ standard. Then, Maxisorp 96-well plates (Nunc) were activated with 100 µg of protein from each fish sample. In addition, the biotinylated-synthetic peptide was used to generate a standard curve from 1 ng/µL to 0.00625 ng/µL in control fish plasma. Each sample was loaded in duplicate. Six wells were activated with 100 µL of phosphate-buffered saline as a control. The microplate was activated overnight at 4°C. Subsequently, the content of each well was removed, and the plate was washed three times with 200 µL of PBS containing 1% Tween 20 (PBS-T). The microplate was then blocked with PBS-T containing 3% bovine serum albumin for 1 hour at room temperature. The blocking solution was removed, and the microplate was washed three times as described above. Subsequently, the streptavidin-peroxidase complex (ThermoFisher) diluted 1:10,000 in PBS was added and incubated with 100 µL at room temperature for 30 min. The solution was removed, and the plate was washed as described above. Finally, 100 µL of Thermo Scientific Pierce 1-Step Ultra TMB ELISA Substrate (Thermo Fisher Scientific) was added to each well and incubated in the dark for 20 min at room temperature. The absorbance of each well at 650 nm was quantified.

2.6 Histochemistry procedure

The tissues were included in paraffin and then were dehydrated ascending alcohol series up to 100 %, and passages were carried out through xylol. The samples continued to be embedded in paraffin, cut at 5 µm and deposited on slides with poly-L-lysine as an adhesion medium. Once the sections were obtained, they were left in an oven at 60°C for better adhesion. The conventional morphological technique of hematoxylin-eosin was applied. The deparaffinization/rehydration with a descending battery of xylols and alcohols were performed once the sections were obtained. The samples were washed in distilled water for 5 min and stained with Harris hematoxylin for 30 seconds. Finally, the histological tissue slides were dehydrated and clarified in a battery of alcohols and xylols, covered with the Entellan™ mounting medium, and covered with a coverslip for later observation in an Olympus CX21 optical microscope.

Biotinylated ghrelin was detected in tissue histological slides using the streptavidin-biotin complex. First, the samples were washed in distilled water for 5 minutes, followed by washing with saline phosphate buffer pH 7.4 (PBS) for 5 minutes. Subsequently, endogenous peroxidase blockage was performed using 30% H_2O_2 (Merck) in PBS for 30 min at room temperature. Then, three washings were performed with PBS for 5 min each, followed by incubation with the streptavidin peroxidase complex (Thermo Fisher) at a 1:200 dilution in PBS/Tween 20 for 1 hour at room temperature. Next, three new washings were performed with PBS for 5 minutes each, followed by incubation with the chromogenic substrate 9-amino-ethyl-carbazole (Vector) for 3 minutes. Next, samples were washed with distilled water for 5 min, and nuclear contrast was performed with Harris hematoxylin for 5 seconds. Subsequently, the samples were dehydrated in an ascending battery ascending alcohol series by rapid steps, each from 95% ethanol to absolute ethanol. Finally, the samples were rinsed in xylol for 5 minutes each, covered with a hydrophobic mounting medium, and covered with a coverslip for subsequent observation under an Olympus CX21 optical microscope.

2.7 Statistical analysis

Data were analyzed for statistical significance using the R version 3.5.2 software. Prior to statistical analysis, all data were tested for normality and homoscedasticity by Shapiro-wilk and Fligner-Killeen test, respectively. Then the basal tissue mRNA expression of Ghre and GHS-R1 were subjected to Krukal-Wallis test. The blood biochemical parameters (glucose), food intake and Ghre mRNA expression after pre-prandial and post-prandial period were analyzed by one-way ANOVA followed by Tukey's mean comparisons. Finally, blood ghrelin levels (synthetic peptide) were analyzed by Mann–Whitney–Wilcoxon test. Differences were considered significant when $P < 0.01$ (**) or $P < 0.05$ (*). Results are represented graphically using GraphPad prism 8.1 as the mean ± standard deviation (SD) of the biological replicates.

2.8. Ethics statement

Fish were maintained and handled following the guidelines of experimental procedures approved by the Ethics Committee of the Universidad Católica del Norte (protocol number CEC_15/2018 with the date of approval 15 November of 2018).

3. RESULTS AND DISCUSSION

3.1 Characterization and comparative analysis of *C. gilberti* ghrelin with teleost homologues.

Experimental evidence shows that environmental variations and production management can affect the biology of fish farmed in intensive systems, such as feed intake. In order to provide a helpful tool for research on the Corvina drum, a new South American aquaculture fish species, this work addressed a characterization of the hormone ghrelin, a molecule described in other fish species, as key in appetite control (Bertucci et al., 2019; Jönsson, 2013).

Firstly, a ghrelin cDNA fragment of 273 bp was obtained from *C. gilberti* contig genome and then confirmed by PCR amplification from stomach samples of juvenile *C. gilberti*. The sequence was deposited in Genbank by the code accession number ON568235. The alignment of the *C. gilberti* ghrelin propeptide sequence and other fish is shown in Figure 1 A. The amino acid sequence of *C. gilberti* ghrelin showed high identity with its homologue of *L. crocea* croaker. The phylogenetic tree supported this result and indicated that *C. gilberti* ghrelin is grouped in the Sciaenidae teleost family. Moreover, the *C. gilberti* ghrelin sequence showed closest phylogenetic relationship with *Dicentrarchus labrax* and *Morone saxatilis*, both belonging to the Moronidae family (Figure 1 B).

The alignment of sequences demonstrates high conservation of the enzymatic cleavage sites in the different teleost species, which give rise to the mature bioactive peptides ghrelin and the peptide associated with ghrelin. After *C. gilberti* propeptide proteolytic processing, a mature ghrelin with 21 residues in length is produced. In the same way, an amidation signal of the carboxyl-terminal end characterized by having a glycine followed by a basic arginine residue is conserved in *C. gilberti* ghrelin (Figure 1 A). Interestingly, amide structure at the C-terminal, firstly identified in ghrelin from the Japanese eel (*Anguilla japonica*), has not yet been seen in mammalian ghrelins (Kaiya et al., 2003b; Kaiya et al., 2008). In addition, non-amidated ghrelin has also been reported in some teleost species, such as rainbow trout (Kaiya et al., 2003a). The physiological significance of ghrelin amidation in their bioactivity in fish remains to be elucidated, but in general the amidated peptides are less sensitive to proteolytic degradation, extending their half-life in the bloodstream (Kumar et al., 2014).

A unique feature of ghrelin mature peptide from mammals to teleost is the modification of Ser-3 with fatty acid, mainly octanoic acid (Kaiya et al., 2008; Kojima and Kangawa, 2005; Nishi et al., 2011). In this way, the N-terminal four amino acids of ghrelin from teleost (including *C. gilberti* ghrelin) is highly conserved (Figure 1 A). These residues were recognized as the "active core" of ghrelin, because it is described as essential for receptor binding (Kaiya et al., 2008).

The ghrelin mRNA expression of *C. gilberti* juveniles was detected in the seven tissues obtained from the preprandial experiment groups, which included spleen, gill, pyloric caeca, stomach, intestine and muscle. Expression levels in all tissues are expressed relative to gill (Figure 2 A). Ghrelin expression was highest in the stomach followed by muscle,

while the lowest expression was found in the liver and spleen ($p>0.05$). This result is similar to the one reported for the *L. crocea* croaker (Liu et al., 2021), closely related to the Corvina drum. However, different levels of ghrelin transcript expression are described in other fish species. For instance, in gibel carp (*Carassius auratus gibelio*), a high ghrelin expression was observed in the intestinal tract, followed by the liver. In contrast, a low expression was noted in the heart, brain, skin, muscles, gill, and eyes (Kitazawa and Kaiya, 2019; Zhou et al., 2016).

As an important regulator of food intake, ghrelin can enhance the sense of hunger before feeding and induce feeding behavior. Following this assumption, a pre and post-prandial experiment with the Corvina drum was performed. The feeding protocol previously established for this species was chosen for the hour of meal delivery. The plasmatic glucose levels confirm the post prandial period, because were significantly higher after being fed (+1 h, $p>0.05$; +6 h, $p>0.01$) when compared to one hour before the first meal (Figure 2 B). Then, to determine whether the pre and post-prandial period influenced the expression of the ghrelin gene in the stomach of *C. gilberti*, cgGhre mRNA expressions in the stomach were compared between pre-prandial and post-prandial fish groups. Gene expression of stomach ghrelin showed a significant increase in its transcript in the pre-prandial period, decreasing after eating food (Figure 2 C) ($p>0.01$). Interestingly, ghrelin levels increased again 6 hours after the first meal ($p>0.05$). The latter would be associated with an increased appetite for the second food ration under culture conditions, which took place during the afternoon.

Among teleosts, the mRNA expression of ghrelin has been analyzed in goldfish (Unniappan and Peter, 2005) Mozambique tilapia, *Oreochromis mossambicus* (Kaiya et al., 2003c), Nile tilapia, *Oreochromis niloticus* (Parhar et al., 2003), rainbow trout, *O. mykiss* (Jönsson et al., 2010), Atlantic salmon, *Salmo salar* (Murashita et al., 2009; Del Vecchio et al., 2021), gibel carp, *Carassius auratus gibelio (*Zhou et al., 2016*)*, grass carp,*Ctenopharyngodon idellus (*Liang et al., 2019*)* and large yellow croaker, *Larimichthys crocea* (Huang et al., 2020). In these teleost fish prolonged fasting periods have significantly up-regulated ghrelin in their stomach or gut (Zhou et al., 2016). In the case of high vertebrate's ghrelin, far greater amounts of DacylGhr than acylGhr exist in the blood daily, where over 90% of the immunoreactivity for ghrelin is for this non-acylated form, both in rats and in humans (Nishi et al., 2011). Remarkably, studies in humans showed that postprandial (two hours after the meal consumption) DacylGhr levels did not change, whereas acylGhr levels decreased (Dardzińska et al., 2014). So, it is likely that the different molecular forms of ghrelin may modulate these sensations differently. Thus, future studies in croaker require evaluating the expression of ghrelin molecular forms under different feeding protocols.

3.2 The acylation increases the orexigenic properties of *C. gilberti* ghrelin

The functional activity of fish ghrelin has been assessed using its acylated-molecular form because different authors assume that the non-acylated ghrelin form is inactive. Here, we

synthetized both molecular forms (Figure 3 A), the acylated (cgGhre) and the deacylated (D-cgGhre) *C. giberti* ghrelin to compare their ability to regulate food intake in *C. gilberti*. As expected, the coupling of octanoic acid in the third serine of N-terminal end increased the peptide hydrophobicity since the fractionation by C18 columns showed that the cgGhre was eluted with a higher percentage of acetonitrile (30%) than D-cgGhre (10%) (data not shown). Circular dichroism (CD) was performed to monitor the relative structural changes caused by the octanoic acid coupling. When the spectra of acylated and non-acylated Ghrelin were compared, it was observed that both displayed characteristics of an unordered conformation with the unique minimum around 200 nm and no maximum (Figure 3 B). Also, it was observed that the peptide with the serine acylation stabilized its unordered conformation. In previous studies, similar results were observed with dermaseptin where the acylation stabilized its structure (Radzishevsky et al., 2005).

Cumulative feed intake analysis showed that the cgGhre further increased food intake by 14% with respect to D-cgGhre ($p>0.05$), and by 39% in the amount of ingested food with respect to the control fish ($p>0.01$) (Figure 4). These results support the idea that incorporating octanoic acid in Ser^3 increases ghrelin bioactivity, which is conserved in teleost ghrelin. Still, it is important to note that the amino acid sequence can increase the croaker appetite because deacylated ghrelin from *C. gilberti* is also capable of significantly increase appetite with 25% more of ingested food than the control fish ($p>0.01$).

Evidence about the function of deacylated ghrelin in teleost fish are relatively scarce. In the widespread vision, deacylated ghrelin does not interact with GHS-R; therefore, it would not have a biological function similar to the acylated counterpart (Hosoda et al., 2000; Zhong et al., 2021). For that reason, some authors describe it as inactive and, therefore, that it does not exert orexigenic action. This is the case of deacylated ghrelin from goldfish, which does not have the ability to induce feed intake (Blanco et al., 2016; Matsuda et al., 2006). In fact, the results of intracerebroventricular or intraperitoneal inoculation of goldfish deacylated ghrelin suppressed the orexigenic action of acyl ghrelin. However, until now, there is no other report comparing the bioactivity of molecular forms of this hormone in fish. Thus, the ability of molecular forms of ghrelin to regulate appetite in fish could be species-specific (Bertucci et al., 2019).

In agreement with our results, the studies in vertebrates reveal that the deacylated ghrelin can up-regulate food intake at a lower level than the acetylated form (Toshinai et al., 2006). Interestingly, the exogenous application of this molecular form increased the expression of neuropeptide Y and orexin, both neuropeptides recognized as potent orexigenic (Toshinai et al., 2006). However, the ability to promote growth hormone synthesis is lost. Due to these results, some authors proposed that deacylated ghrelin should be regarded as a different hormone than acyl ghrelin because it could induce food intake by a mechanism independent of the growth hormone secretagogue receptor (Toshinai et al., 2006; Zhong et al., 2021). However, no specific receptor for this molecular form of ghrelin has been reported; therefore, the potential role and mechanisms of action and functional significance of deacylated ghrelin remain to be established (Fernandez et al., 2016). This is even less

known in teleost fish, so it is necessary to continue advancing in dose-response studies to evaluate the functional properties of both molecular forms of ghrelin in sciaenids and other teleost fish.

3.3 Ghrelin uptake and distribution in the gastrointestinal tract of *C. gilberti*.

Since the application of acylated ghrelin presented the highest orexigenic capability, we decided to continue exploring whether this peptide can be absorbed and distributed to the gastrointestinal tissues from *C. gilberti*. To do so, the synthetic peptide was coupled to biotin; therefore, the non-use of antibodies against the molecule allows following the exogenously applied peptide through the biotin-streptavidin complex reactivity.

The plasma values of synthetic cgGhre 2 hours post-injection (hpi) showed a high blood concentration deviation with Corvina drum containing 0.69 ng/dL and others containing 0.34 ng/dL, with a mean of 0.24 ng/dL (Figure supplementary 1). Control fish injected with peptide vehicle (saline teleost solution) showed no absorbance for biotin-streptavidin complex in fish plasma (data not shown), while the plasma ghrelin levels from fish 8 hpi decreased to a range between 0.03 and 0.19 ng/dL, with a mean of 0.12 ng/dL. Although there is a clear tendency to decrease the amount of peptide after 8 hours of being supplied, no significant differences were obtained between the measurement of blood ghrelin from the fish group at 2 hpi with those of the group at 8 hpi ($p=0.0571$).

Most studies have focused on the orexigenic and/or somatotropic pathways of ghrelin; therefore, little is known about the kinetics of this peptide in different animal models. Here, the data of plasma ghrelin indicates an extended half-life of the peptide in the blood circulatory system of teleost, which could be associated with the increasing peptide stability by amino-end amidation (Kumar et al., 2014). However, this does not imply that the peptide cannot undergo modifications, such as deacylation. For instance, the *in vitro* half-life of octanylated ghrelin immunoreactivity in plasma of mice showed deacylation at 12.8 min (Hiejima et al., 2009). Thus, future studies should be conducted to assess if the peptide could be enzymatically modified in the fish blood. Likewise, this strategy showed that the peptide could be absorbed and distributed through the blood into different fish tissues. In this study we focused on digestive tissues of Corvina drum.

In juvenile and adult fish, the gastrointestinal system shows great diversity in form and function, depending on feeding habits, which in many cases means efficient primary digestion and a large intestinal absorptive surface area (Ray and Ringø, 2014). The Corvina drum has a short digestive tract (Figure 5 A), typical of carnivorous fish, which is shared by the morphology described for other croakers in the sciaenidae family (Kalhoro et al., 2018; Shan et al., 2016). Following the common pathway for the absorption and distribution of compounds applied in fish and other animal models, the biotin-labeling ghrelin was first evaluated in the liver. In the case of *C. gilberti*, the exocrine pancreatic tissue is diffusely

set inside the liver (called hepatopancreas); for this reason, pancreatic acini are visualized in the section together with hepatocytes (Figure 5 B). The histochemistry analysis of biotin-streptavidin reactivity identified cgGhre-positive cells from hepatopancreas only after 2 hpi of the synthetic peptide (Figures 6 A and B). Furthermore, the reactive cells were localized close to the blood vessel, which suggests the hepatic uptake of the synthetic peptide and its entering into blood circulation (Figures 6 A and B). In contrast, no cgGhre reactivity after 8 hpi was observed (Figures 7 A and B). These results are supported by the cgGhre plasmatic measurement showing the highest peptide concentration at 2 hpi. Therefore, the peptide could be metabolized and distributed within the first hours after injection.

Different research reports have demonstrated the direct relationship between lipophilicity of small targeting peptides and hepatic uptake and clearance (Hosseinimehr et al., 2012; Krenning et al., 1992; Lin et al., 2004). Then, ghrelin acylation possibly helps its absorption and distribution from the liver into the fish tissues.

The stomach is the main organ associated with the synthesis of ghrelin, but it is also where the peptide has the ability to modulate the tissue function as an orexigenic factor (Du et al., 2016). Our results support the latter, since after 2 h of peptide application positive cgGhre reactivity was observed preferentially located in areas close to the lumen of the fundus region of the stomach (Figures 6 D and E).

A direct relationship between ghrelin and digestive enzymes has been reported in mammals, but appears to be species-specific because acylGhr increases pepsin activity in gastric mucosal cells of piglets (Du et al., 2016) and the activity of lysosomal hydrolases in rabbits (Witek et al., 2005), although it reduces the volume of pancreatic-biliary juice protein and trypsin outputs in rats (Kapica et al., 2008). In fish, only one report describes ghrelin's ability to regulate the digestive enzymes in goldfish (Blanco et al., 2017). The work described an interaction between ghrelin and some key digestive enzymes secretion in the intestine and hepatopancreas explants cultures. The most notable effect of ghrelin is on sucrase-isomaltase, suggesting a more important role for ghrelin in modulating this particular enzyme in this fish species. In addition, the use of a receptor antagonist provided evidence that the GHS-R1a mediates such ghrelin actions (Blanco et al., 2017). In this way, the mRNA of GHS-R1 is highly expressed in the *C. gilberti* stomach (Figure 8).

Once again, the large amount of ghrelin found in the submucosa of Corvina drum pyloric caeca attracts our attention (Figures 6 G and H). As in the stomach, a high positive reactivity was found in this tissue 2 hours after the compound was injected. However, unlike what was observed in the stomach, the peptide was not localized 8 hpi intraperitoneally (Figures 7 H and I). Further studies will be required to deepen on the orexigenic actions that ghrelin could carry out in this tissue. Nevertheless, the first insight about the levels of GHS-R1 expression in pyloric caeca from *C. gilberti* suggests a regulatory function in this tissue (Figure 8).

The biological activity of ghrelin stems from its ability to bind and activate GHS-R receptors, described as growth hormone secretagogue receptors (Kaiya et al., 2010). Two GHS-R have been described in vertebrates: one is a functional G-protein-coupled receptor

with seven transmembrane domains, named GHS-R1a, whereas the other is a truncated form of GHS-R1a lacking transmembrane domain 5, named GHS-R1b (Albarrán-Zeckler and Smith, 2013). Different authors reported these GHS-R isoforms in teleost fish, such as black seabream, goldfish, and zebrafish (Blanco et al., 2016; Kaiya et al., 2013, 2010; Perelló-Amorós et al., 2019; Small et al., 2019). In addition, the presence of another GHS-R, named GHS-R2a, was reported in zebrafish, channel catfish, and goldfish, showing amino acid sequence similarity with GHS-R1a (Kaiya et al., 2010; Small et al., 2019). According to gene expression analysis, the GHS-R mRNA is produced in many tissues of Corvina drum fish, including those critical in peripheral and central feeding regulation, e.g. brain, liver, and gastrointestinal tract (Figure 8).

The functionality of the GHS-R of teleost fish has been confirmed through the quantification of intracellular Ca^{2+} when receptor-transfected human cells are stimulated by catfish ghrelin and by the use of ghrelin antagonist ([D-Lys3]-GHRP-6 (Nisembaum et al., 2014; Small et al., 2019). Thus, future studies will be focused on the functionality of molecular forms of ghrelin in the ability to activate GHS-R in these tissues from *C. gilberti*. The findings presented here support the idea that ghrelin may assist peripheral orexigenic function in the digestive tissues of croakers.

4. Conclusions

The purpose of this study was to identify and characterize the ghrelin orexigenic peptide in the *C. gilberti*. For the first time, we have revealed that ghrelin peptide sequence is highly conserved between teleost fish, especially in the active core in the N-terminal end. Ghrelin from *C. gilberti* is highly expressed in the stomach and lesser extent in others tissues of gastrointestinal tract suggests that the ghrelin could be involved in a variety of physiological functions in the digestive process of these organs. After observing the expression profiles of mRNA ghrelin during the pre and post-prandial periods, we conclude that ghrelin may be an appetite-inducer in this species. Moreover, our results from the *in vivo* studies confirm the orexigenic bioactivity, indicating that the molecular forms of ghrelin increase feed uptake and locally interact with digestive tissues in *C. gilberti*. This first study with appetite regulatory peptides from Corvina drum provides a basis for future research about the regulating mechanisms and functions that trigger the role of orexigenic peptides in sciaenid aquaculture species.

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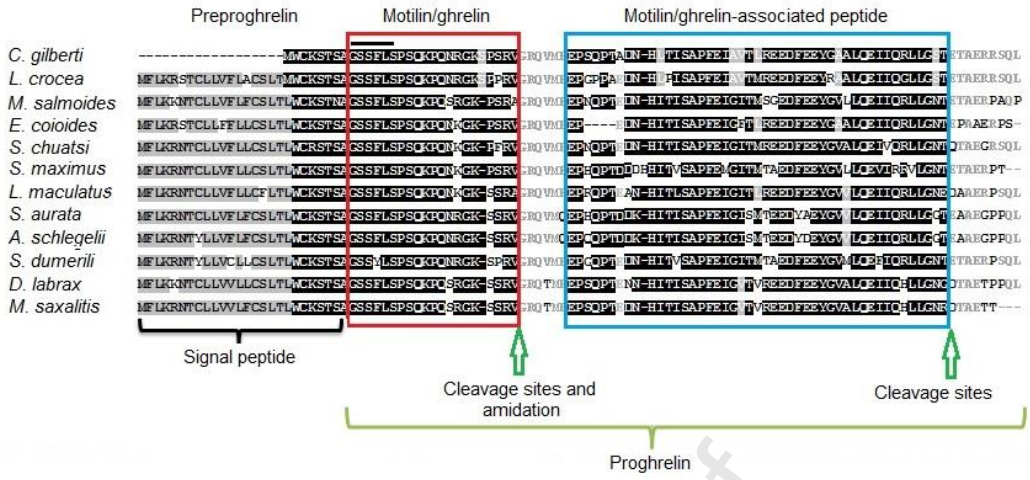

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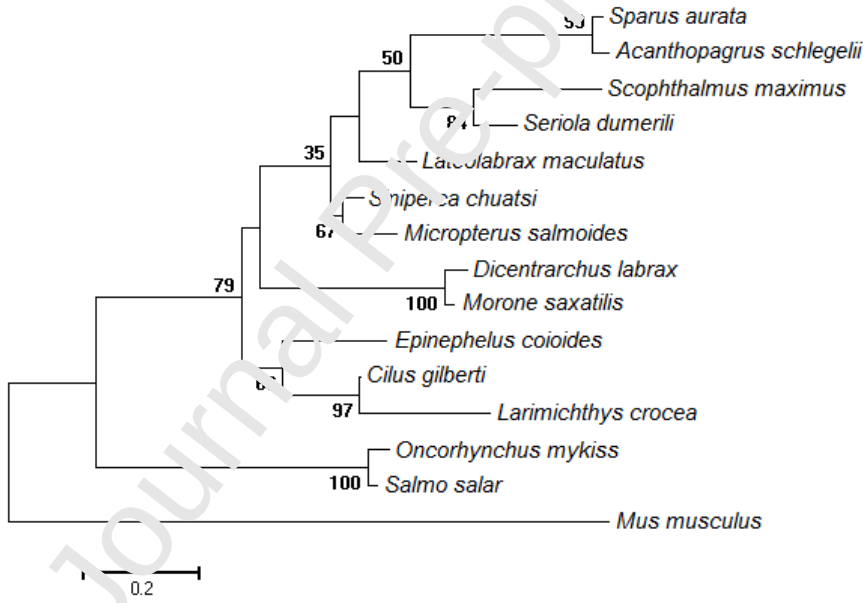

Figure 1. Characterization of ghrelin propeptide sequence from *C. gilberti*

A) Multiple alignment of ghrelin amino acid sequences from teleost species. Identical amino acids are highlighted in black and printed in white letters. Putative cleavage sites are shown by green arrows. The mature peptides after propeptide processing are indicated by red and sky-blue lines. **B)** Phylogenetic tree of the ghrelin for Bayesian phylogenetic inference using MCMC methods with likelihood model. The percentage of replicate trees in which the associated taxa clustered together in the 10,000 replicates are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the

evolutionary distances used to infer the phylogenetic tree. Each branch to contain the GenBank accession number corresponding to the sequences used for the reconstruction next to species name and (1 or 2) designating to the homologous variant.

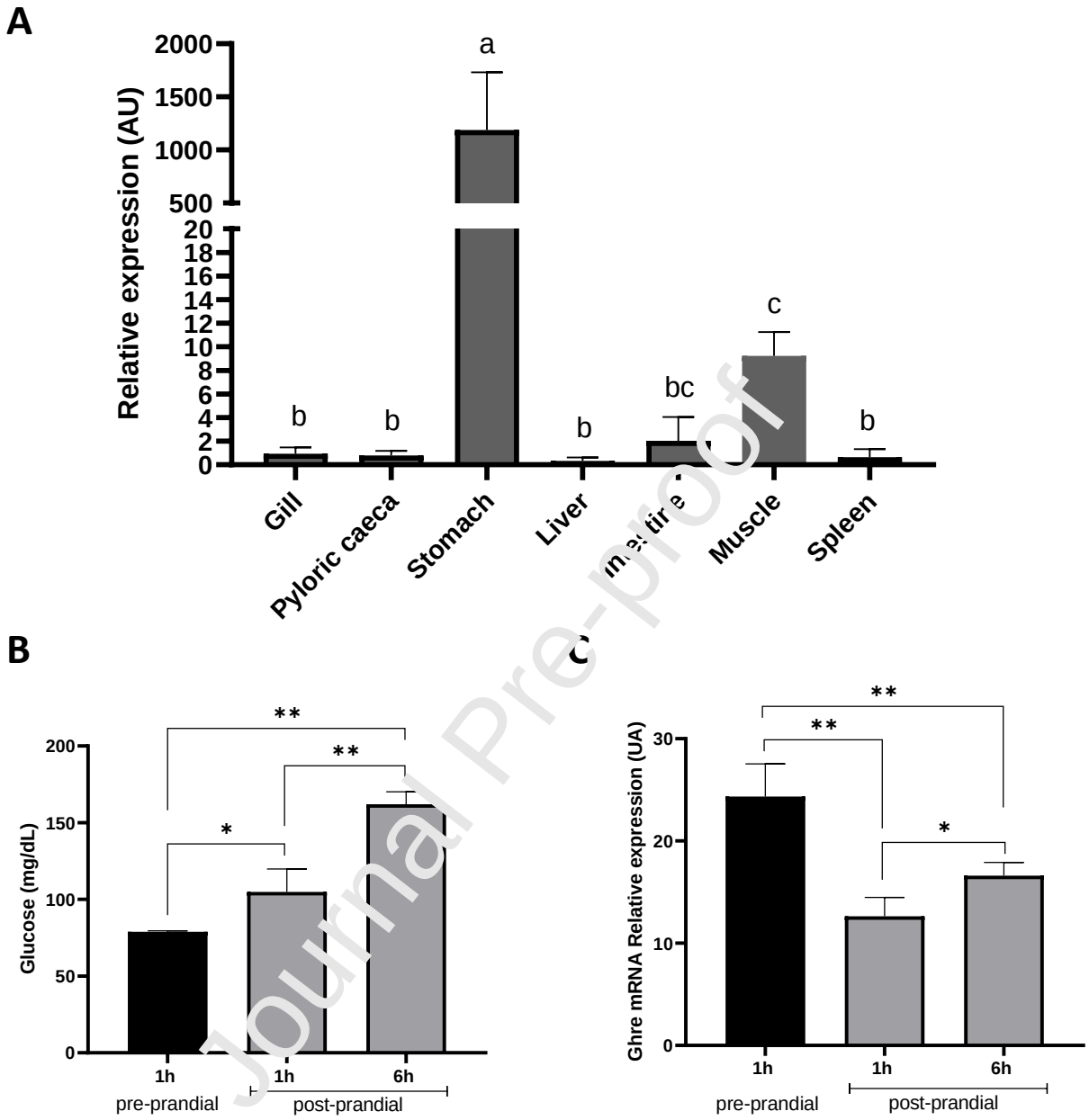

Figure 2. Basal tissue mRNA expression of ghrelin from *C. gilberti* and effects of feeding period.

A) Ghrelin mRNA expression in *C. gilberti* tissues are expressed as relative gene expression with β-actin as normalizer. Data are given as mean ± SD and differences were considered significant when $P < 0.05$ and indicated with a different letter (n=6). **B)** Levels of plasmatic glucose from *C. gilberti* fish group of pre-prandial and post-prandial period (n=8). **C)** Ghrelin mRNA expression in *C. gilberti* stomach from fish group of pre-prandial and post-prandial period. For B and C, data are given as mean ± SD and differences were considered significant when $P < 0.01$ (**) or $P < 0.05$ (*), n=8.

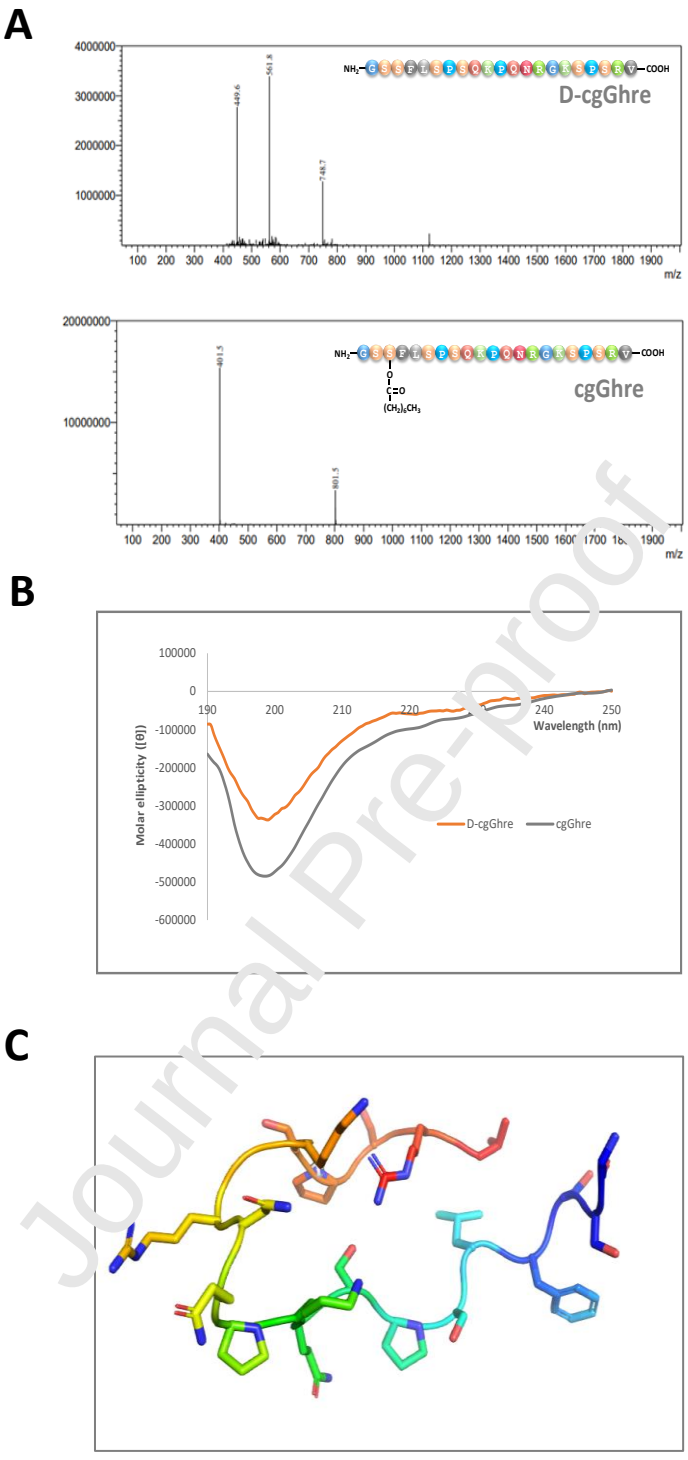

Figure 3. Chemical synthesis and peptide structure of *C. gilberti* ghrelin.
A) ESI-MS spectrum of D-cgGhre (top) and cgGhre (below), showing the charged ion mass of synthetic peptides. In addition, the peptide sequence in each case is shown. **B)** Circular dichroism of D-cgGhre and cgGhre synthetic peptides in 30% trifluoroethanol. CD spectra

was recorded in the far UV and expressed in molar elipticity (θ). **C)** Representative 3D model of cgGhre.

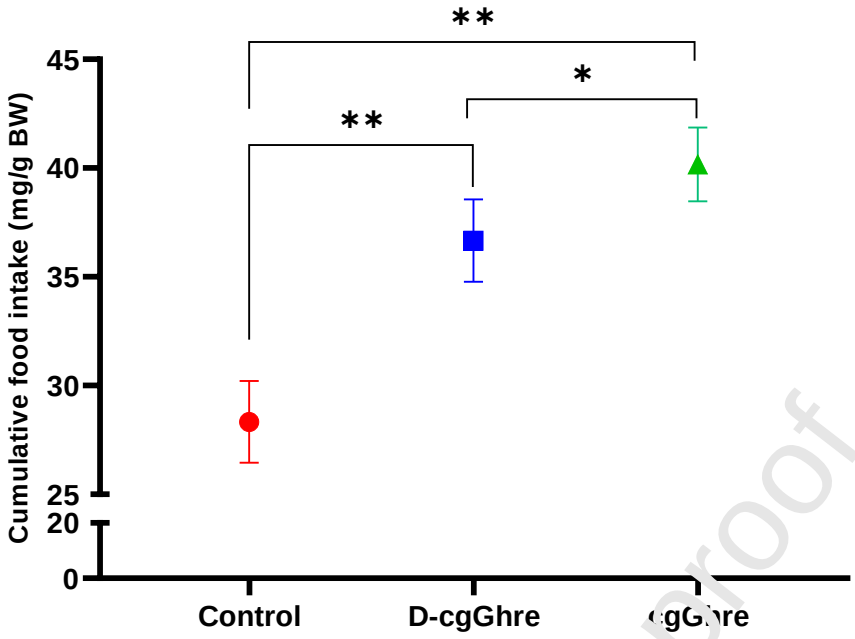

Figure 4. Effect of ghrelin in the food intake of *C. gilberti* juveniles.
Cumulative food intake of juveniles from *C. gilberti* was determined after intraperitoneal injection of 100 ng/g BW of D-cgGhre (blue) and cgGhre (green) suspended in sterile 0.65% NaCl (teleost saline). Fish of control group received IP injection of the same volume of teleost saline (red). The unconsumed feed was used to analyze the food intake expressed as milligrams of food consumed per gram wet BW (mg/ g BW). Data are given as mean ± SD. Differences were considered significant when $P < 0.01$ (**) or $P < 0.05$ (*), n=4.

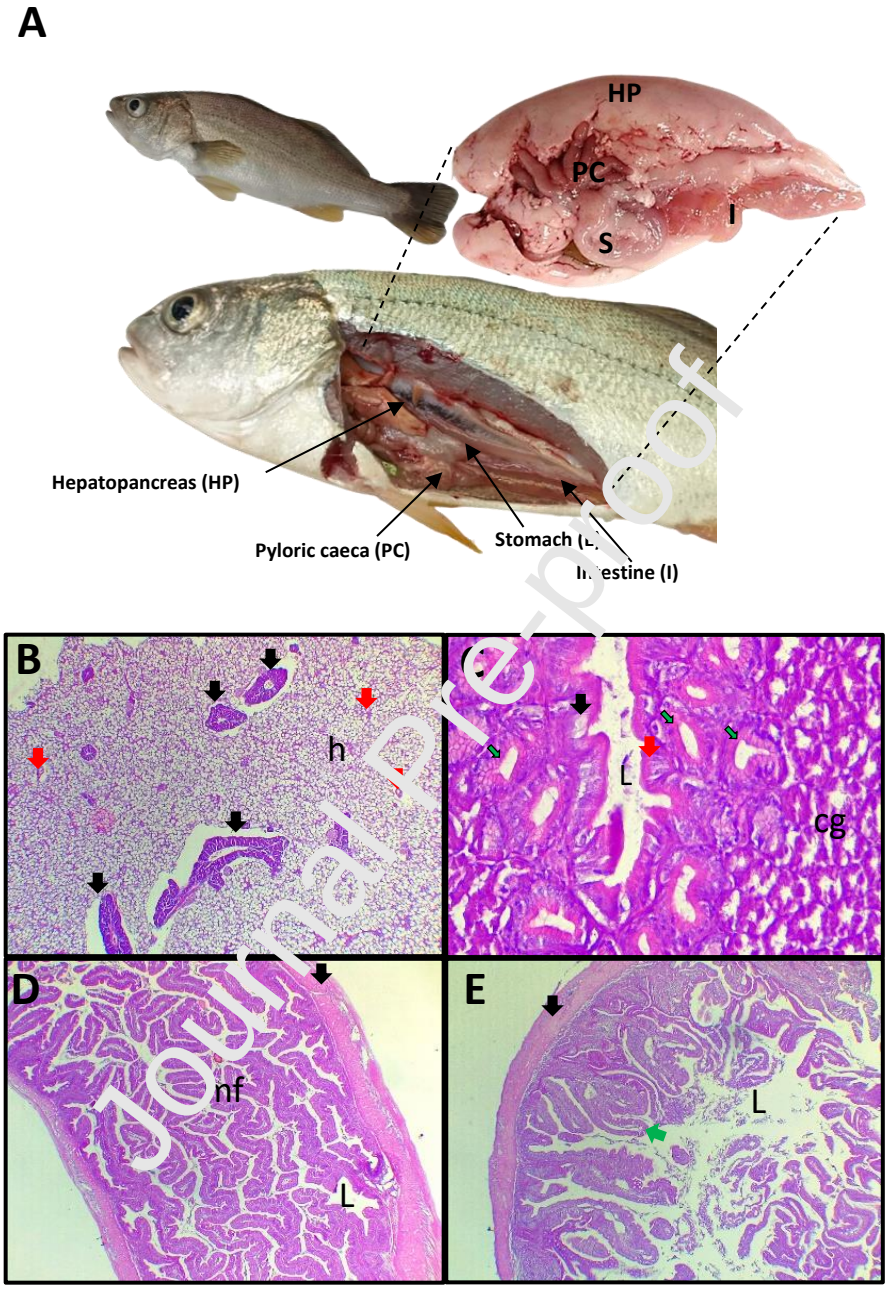

Figure 5. Characterization of digestive tissues from *C. gilberti* juveniles.
A) Representative images of anatomic location of hepatopancreas, pyloric caeca, stomach and intestine of *C. gilberti* juveniles with 150 grs in total body weight. **B)** Histological slide of hepatopancreas stained with hematoxylin and eosin stain showing the acini pancreatic

(black arrow) diffusely set inside of liver (h). In addition, the liver blood vessels are indicated by red arrows. **C)** Histological slide of stomach stained with hematoxylin and eosin stain showing the mucous secreting cells (green arrows), gastric gland (black arrows), simple columnar epithelium (red arrows) and the lumen (L) and cardiac gland (cg). **D)** Histological slide of a longitudinal section of the pyloric caeca with a labyrinthine aspect stained with hematoxylin and eosin stain showing mucosal folds (mf), lumen (L) and muscularis (black arrows). **E)** Histological slide of a longitudinal section of the intestinal mucosa stained with hematoxylin and eosin stain showing epithelium (red arrows), lumen (L) and muscularis (black arrows).

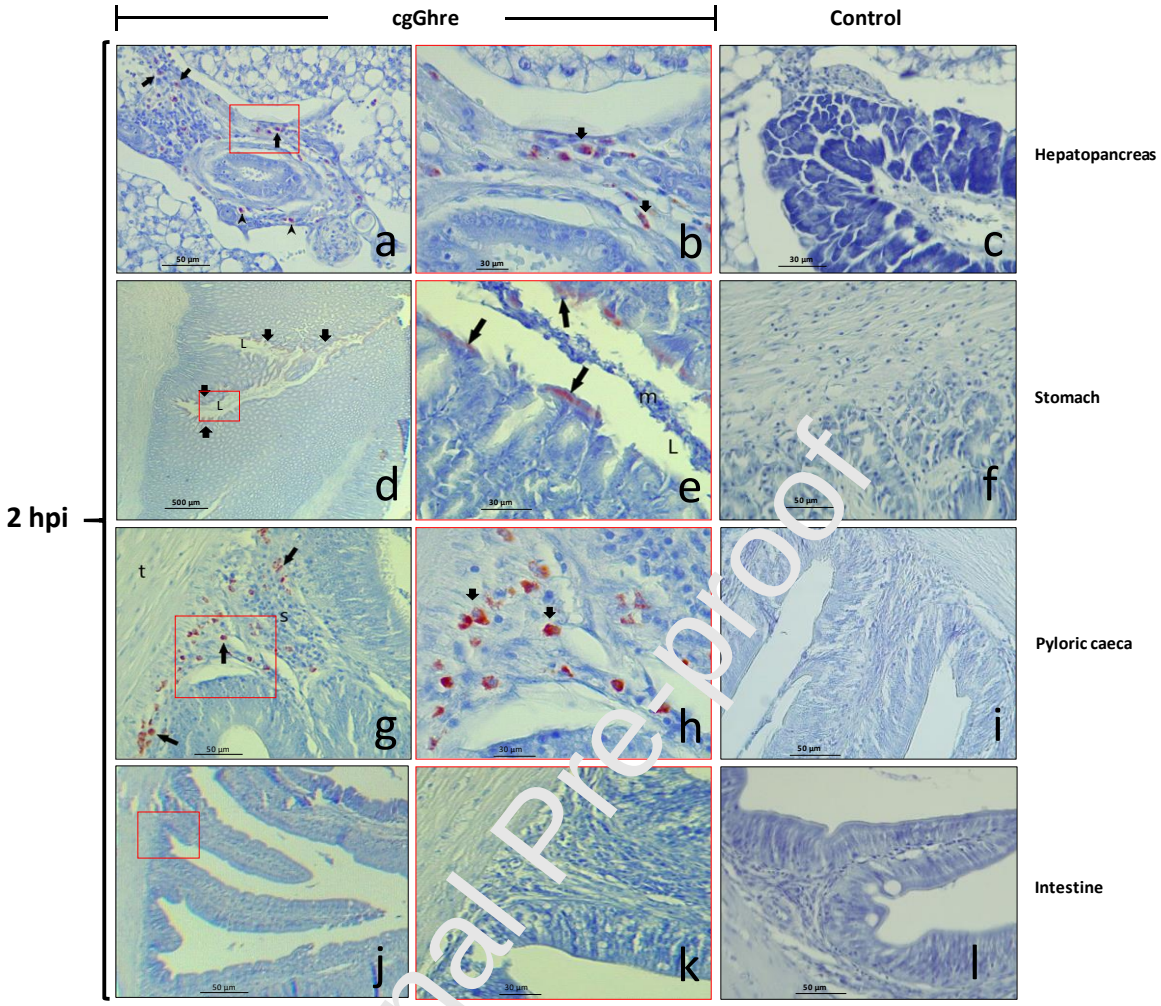

Figure 6. Location of synthetic ghrelin in the digestive tissues from *C. gilberti* juveniles after 2 hour post injection.

Representative images of histochemical detection of biotinylated cgGhre on hepatopancreas, stomach, pyloric caeca and intestine from juveniles *C. gilberti* after 2 hours post intraperitoneal injection (hpi) of synthetic peptide. Control fish were injected with saline teleost (n=4). Red square indicates amplified area in each tissue. Black arrows indicate positive reactivity of cgGhre in each tissue histological slides. The lumen (L) and the mucus (m) of stomach is indicated in d and e micrographs. In addition, the submucose zone (S) in the pyloric caeca slide is indicated in micrograph (g).

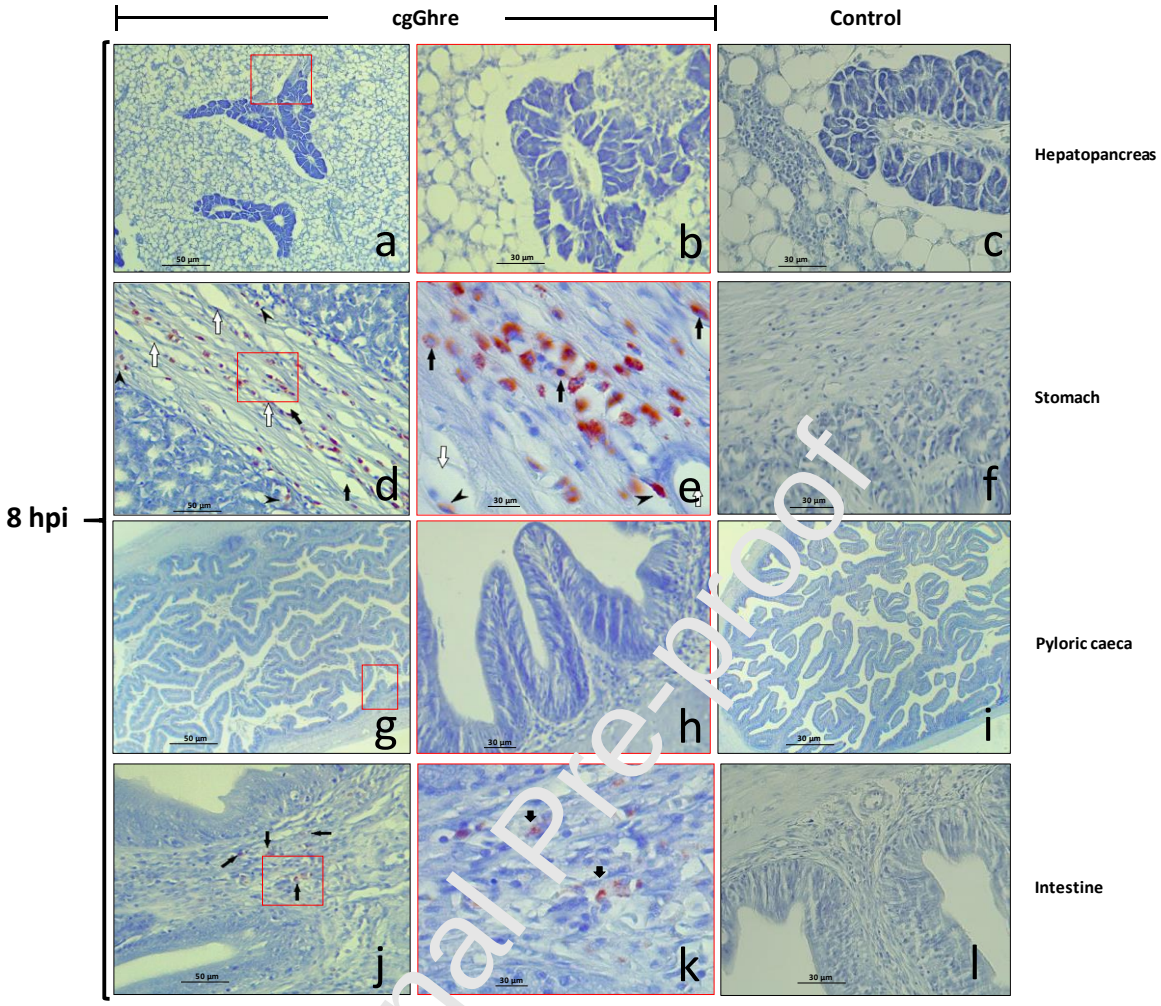

Figure 7. Location of synthetic ghrelin in the digestive tissues from *C. gilberti* juveniles after 8 hours post injection.

Representative images of histochemical detection of biotinylated cgGhre on hepatopancreas, stomach, pyloric caeca and intestine from juveniles *C. gilberti* after 8 hours post intraperitoneal injection (hpi) of the synthetic peptide. Control fish were injected with saline teleost (n=4). Red squares indicate amplified area in each tissue. Black arrows indicate positive reactivity of cgGhre in each tissue histological slides. White arrows indicate stomach blood vessels in d and e micrographs.

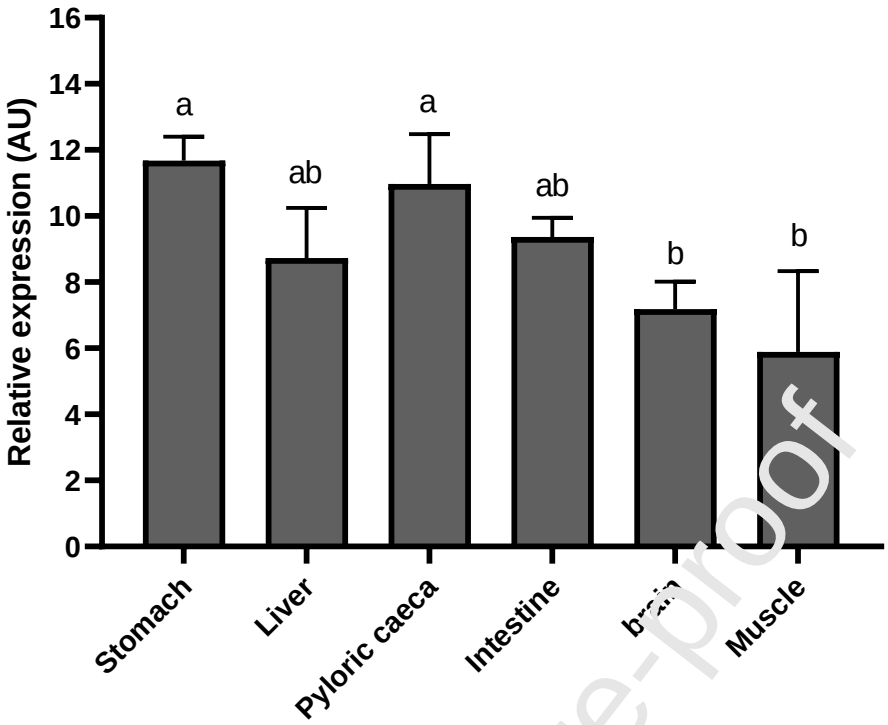

Figure 8. Basal tissue mRNA expression of GHS-R from *C. gilberti*

A) GHS-R mRNA expression in *C. gilberti* tissues are expressed as relative expression with β-actin as normalizer. Data are given as mean ± SD (n=6). Differences were considered significant when $P < 0.05$ and indicated with a different letter.

Author Agreement Statement

We the undersigned declare that this manuscript is original, has not been published before and is not currently being considered for publication elsewhere. We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us. We understand that the Corresponding Author **Claudio A. Alvarez, PhD** is the sole contact for the Editorial process. He/she is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proof

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and the project was run as my

- M.Sc. project

This research project was conducted from 1/11/2018 to 30/10/2022
Starting date Ending date

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Highlights:

- The orexigenic ability of ghrelin was demonstrated in juveniles of *C. gilberti.*
- Ghrelin levels in the stomach increased in the preprandial period
- Synthetic ghrelin was localized mainly in the stomach and pyloric caeca
- Ghrelin is a orexigenic regulator of gastrointestinal tract of *C. gilberti*