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Characterization and gene expression profiles of *Cilus gilberti* leptin in response to culture parameters

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A R T I C L E I N F O

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A B S T R A C T

The Chilean meagre, *Cilus gilberti*, emerges as a novel species to South American aquaculture. Nevertheless, the successful establishment of its cultivation necessitates careful consideration of productive parameters and tolerance to abiotic stressors. To address these challenges, the identifications of biomarkers emerges as a promising approach. Indeed, leptin stands out as a potential biomarker, given its multifaceted role. This study focuses on characterizing leptin in *C. gilberti* (cgLep) and assessing its gene expression in juveniles exposed to different culture and feeding conditions. The results indicated that cgLep coding sequence yields a 137-amino acid prohormone, preserving high relevant cysteine residues for maintaining its structural integrity. Basal cgLep gene expression profiles reveal its expression mainly in the liver, followed by white muscle. Conversely, its receptor (cgLepR) exhibits higher levels in white muscle. Additionally, it was observed that juveniles in a postprandial state exhibited an upregulation of hepatic cgLep. Simultaneously, the cgLepR showed a significant increase at brain level during the preprandial stage. The exposure to acute hypoxia revealed a notable upregulation of cgLep after two hours of low oxygen availability and returned to normoxia baseline after six hours post-hypoxia. Notably, when subjected to a prolonged daily hypoxia regimen, no significant differences in leptin mRNA expression were observed. This suggests a dynamic adaptive response of the *C. gilberti* leptin system to hypoxia. Finally, juvenile Chilean meagre cultivated at 15 Kg/m^3 showcase significant individual variability in hepatic leptin expression levels when compared to those cultured at 25 or 35 kg/m^3. These observations highlight that leptin primarily expresses in the liver and its expression is notably influenced by fasting periods and chronic stress induced by low stock density. This variability in leptin expression could potentially impact food intake, emphasizing the importance of considering these factors in the design and implementation of aquaculture practices for Chilean meagre.

1. Introduction

Leptin, a pleiotropic hormone found in both mammals and fish, plays a pivotal role in regulating broad spectra of physiological processes, including appetite, lipid metabolism, growth, reproduction, stress, and immune response. Despite its role in vertebrates, the functional properties of leptin exhibit intriguing divergences among teleosts, suggesting a species-specific adaptation that may be intricately linked to variations in feeding behaviors and environmental habitats (Friedman, 2015; Deck et al., 2017).

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In its precursor form, known as the propeptide, this protein lacks glycosylations, exhibits high hydrophilicity, and features a 21-amino acid amino-terminal secretory signal, which is removed prior to its release into the bloodstream. Notably, in pufferfish, the propeptide is composed of 137 amino acids, 15 amino acids fewer than its mammalian counterpart. Although it shares only 13 % identity with the amino acid sequence of human leptin, crucial residues essential for both its activity and structural integrity are conserved in the pufferfish propeptide (Vissers et al., 2013). In mammals, leptin synthesis and release performed mainly by adipose tissue, responding to the levels of fat deposits. Conversely, in teleost fish, the liver has been described as the primary site of leptin production (Blanco and Soengas, 2021). Nevertheless, the transcript synthesis capacity of leptin is also documented in various tissues, including the brain, gonads, muscle, and kidney. These tissues exhibit distinct expression profiles, highlighting interspecies variations in leptin distribution and function (Won et al., 2012).

Leptin exerts its action on different tissues, through recognition by its receptor (LepR), also known as ObR, due to its relationship with obesity in humans (Shaikh et al., 2018). Remarkably, in fish, the presence of leptin isoforms with heightened affinity for its receptor has been documented. Investigations involving zebrafish (*Danio rerio*) and Japanese rice fish (*Oryzias latipes*) have demonstrated that isoform A exhibits a superior binding affinity to the receptor compared to isoform B (Prokop et al., 2012). Consequently, research exploring the characterization and functional roles of leptin in newly studied fish species concentrates on leptin A isoform.

Leptin, through its receptor interaction, triggers the modulation cellular function in different tissues via JAK/STAT signaling pathway activation (Janus Kinase / Signal Transducer an Activator of Transcription) (Hsuchou et al., 2009). This cell modulation leads to an alteration in the expression of target genes within tissues expressing the receptor, contributing to the pleiotropic actions of leptin (Hyeong-Kyu and Rexford, 2014).

While the endocrine mechanisms regulating appetite in teleosts are similar to those in mammals, there is limited information on how stressors affect leptin expression and its relationship to food intake. This knowledge gap is especially important in aquaculture-relevant species, where intensive production practices often expose them to prolonged stress episodes, such as during transportation, fluctuations in environmental parameters, changes in feeding regimes, or alterations in stock density. In fact, several studies have provided initial evidence regarding the impact of specific stressors on peripheral endocrine signals that regulate food intake (Sun et al., 2017). Stocking density has a direct impact on food intake in fish, and this holds particular significance in aquaculture. Various fish species exhibit aggressive behavior leading to the emergence of "dominant individuals" and "subordinates", with notable differences in feeding behavior between these two groups (Rønnestad et al., 2017). Additional studies have explored various markers of appetite concerning culture density, suggesting that beyond the integration of peripheral and central signals in the hypothalamus, there is a significant role played by peripheral endocrine signals of leptin. These insights emphasize the intricate interplay between social or environmental factors and the regulatory mechanisms governing appetite in commercial fish species (Conde-Sieira et al., 2018). For instance, Álvarez et al. (2023) observed increased Leptin gene expression in the liver of *Salmo salar* at the highest density evaluated (40 Kg/m3), compared to the control group of 11 Kg/m3 after 21 and 40 days of culture. This finding highlights the potential influence of culture density on leptin expression and suggests a dynamic relationship between stocking density and the molecular regulation of appetite-related genes in aquaculture fish species.

A growing concern for the global aquaculture industry is the diminishing availability of dissolved oxygen (DO), a stress factor exacerbated by climate change. This phenomenon poses an escalating threat to aquatic systems, leading to heightened instances of hypoxia in terms of both intensity and frequency. These hypoxic episodes are particularly problematic in areas worldwide where marine farms are situated, adding an additional layer of challenge to the aquaculture sector. Addressing the impact of low dissolved oxygen levels is becoming increasingly imperative as climate change continues to influence the dynamics of aquatic environments (Pollock et al., 2007). Indeed, low oxygen levels have been linked to appetite suppression, a common response observed in fish experiencing hypoxia. Bernier et al. (2012) conducted a study with common carp (*Cyprinus carpio*), exposed to 10 % O_2 saturation for eight days, resulting in a substantial 79 % reduction in feed consumption compared to fish maintained under normoxic conditions. The study suggests that appetite suppression in response to chronic hypoxia may be attributed to the modulation of anorexigenic signals. Notably, the authors also found that hepatic leptin expression increased in individuals subjected to chronic hypoxia. This underscores the intricate relationship between low oxygen levels, appetite regulation, and the potential involvement of leptin in mediating physiological responses to hypoxic stress in fish (Bernier et al., 2012).

In teleost fish, there is stronger evidence establishing a direct correlation between stress events and alterations in food intake. Recognizing and delineating molecular markers that interconnect these two processes are imperative for standardizing various culture parameters in fish farming. This becomes especially critical for species undergoing productive scaling, as is the case with *Cilus gilberti*, which is actively included in aquaculture diversification programs in Chile and Peru. The initial productivity outcomes highlight *C. gilberti* as a promising marine species for high-density cultivation. Notably, the species exhibits enhanced growth capacity at densities exceeding 35 Kg/m^3 (Álvarez et al., 2020). However, a comprehensive examination of whether these conditions exert broader impacts on diverse physiological processes is imperative. Identifying and understanding the molecular markers associated with stress and food intake in *C. gilberti* is pivotal for optimizing its aquaculture practices, contributing to the success of breeding initiatives, and ensuring sustainable production in the context of expanding aquaculture operations. To address this need, our study focuses on characterizing leptin and its receptor in *C. gilberti*. We aim to determine their transcription levels in tissue samples obtained from one-year-old *C. gilberti* juveniles during both pre- and post-prandial periods. Moreover, we investigate their expression under different culture densities and in response to acute and chronic hypoxia events. This comprehensive molecular analysis will provide valuable insights into the intricate interplay between leptin signaling, fasting state and the physiological responses of *C. gilberti* to varying environmental and culture conditions.

2. Material and methods

2.1. Fish maintenance and bioassays

The research employed *C. gilberti* juveniles sourced from Fundación Chile facilities located in Tongoy Bay, Coquimbo, Chile. After that, the juveniles were transported to the Fish Culture Laboratory at Universidad Católica del Norte in Coquimbo. Throughout the acclimatization phase, the fish were housed in 1 m^3 tanks, with daily monitoring of temperature (16.5 ± 0.5°C), dissolved oxygen (DO) of 7.5 mg/mL ± 0.5 using multiparameter equipment (Hanna) and a photoperiod of 12:12 (L/D). A feeding regimen consisting of a 1 % biomass proportion was employed, with two daily rations supplemented manually.

The animal study protocols were approved by the Ethics Committee of Universidad Católica del Norte Sede Coquimbo (CEC UCN Nº 01 12th May 2020 and CEC UCN Nº 05 22th March 2023).

2.1.1. Acute hypoxia bioassay

For this bioassay, thirty six *C. gilberti* juveniles with an average total mass of 64.5 g were utilized, and they were evenly distributed among six 40 L tanks. Three tanks maintained normoxic conditions with an DO of 7.5 ±0.5 mg/mL, while the remaining three tanks were subjected to an

acute hypoxia event. During the hypoxia treatment, the air supply to the tanks was stopped, and nitrogen (N_2) was introduced into the water until the oxygen concentration dropped to 2.0 ±0.5 mg/mL for a duration of 2 hours. Subsequently, air was reintroduced until the normoxic condition was restored. Throughout the study, the temperature in each tank was rigorously maintained at 16.5 ±0.5°C.

After exposure to hypoxic conditions for two and six hours, three fish were randomly selected from each tank. Simultaneously, three individuals were chosen from each tank under normoxic conditions. These fish were placed in containers containing an anesthetic overdose solution (200 mg/L of Tricaine MS-222 in seawater). Body mass (g) and length (mm) measurements were recorded for each fish, and a blood sample was obtained from the tail vein using heparinized syringes. The samples were then centrifuged at 5000 x g for 5 minutes at 4 °C, and the plasma fraction was stored at −20 °C.

Various tissue samples (including spleen, gills, brain, pyloric caeca, stomach, liver, foregut, hindgut, white muscle, anterior kidney and posterior kidney) were extracted from each fish and promptly placed in two sterile tubes containing RNAlater® (Thermo Fisher) for preservation. These samples were then stored at −80°C for further analysis.

2.1.2. Intermittent hypoxia bioassay

The samples used in this study were obtained as reported from a previously published bioassay conducted by our research group (Vega et al., 2024). In summary, a total of 200 juveniles, averaging of total mass of 210 g each, were randomly distributed to eight conical 250 L tanks. This distribution maintained 25 fish per tank in seawater within an open-flow system, ensuring a stable temperature of 16.5 ± 0.5°C and sufficient aeration. Following a two-week acclimatization period, the fish were divided into two groups, each comprising four tanks.

The first group, serving as the control, remained under normoxic conditions (7.8 mg/L dissolved oxygen) for the duration of the experiment. Meanwhile, the second group was subjected to daily hypoxic events (2 mg/L dissolved oxygen) lasting 3 hours each, over a span of 30 days. Hypoxic conditions were induced by temporarily halting the water and air flows and injecting nitrogen (N_2) into the water.

At intervals of 0, 15 and 30 days into the experiment, liver samples were collected from both the control and hypoxia-exposed groups using the previously described methods.

2.1.3. Pre-postprandial bioassay

The samples used in this study were obtained from a previously published bioassay conducted by our research group (Álvarez et al., 2023). In summary, a group of 40 juveniles with an average total mass of 95 g were divided into four 80 L tanks, with two tanks designated for preprandial sampling and the other two for postprandial sampling. Prior to the bioassay, all fish underwent a fasting period of 48 hours.

Preprandial sampling involved sacrificing three fish per tank one hour before the commencement of the first daily meal (at 08:00). Postprandial sampling occurred by removing three fish per tank one hour and six hours after the initiation of the first meal. Fish selected for sampling were euthanized using an anesthetic overdose (200 mg/L of Tricaine MS-222 in seawater). Body weight (g) and length (mm) were recorded from the sedated fish, along with a blood sample collected from the caudal vein using 1 mL heparinized syringes. These blood samples were processed as previously described to obtain plasma. Additionally, samples of the specified tissues were collected for further analysis.

Plasma concentrations of glucose and triglycerides (TAG) were determined using commercial kits from Spinreact (Spain), following the manufacturer's protocols. Total protein concentration was measured utilizing the Pierce BCA Protein Assay Kit (ThermoFisher Scientific).

2.1.4. Stock density bioassay

The liver samples utilized in this study were sourced from a previous investigation conducted by our research group (Álvarez et al., 2020). In summary, juvenile *C. gilberti* (n=2000; average weight 125 ± 15 g) were procured from Fundación Chile facilities located in Tongoy Bay, Coquimbo, Chile, and subsequently relocated to the Fish Farm Laboratory at Universidad Católica del Norte in Coquimbo.

These juveniles were kept in circular fiberglass tanks (1.3 m^3) equipped with oxygen pumps, biological filters, ultraviolet sterilizing units, and a continuous flow of seawater. They received feedings three times a day (at 09:00 AM, 14:00 PM, and 17:30 PM) manually, beginning in January and continuing until the start of February 2017, serving as an adaptation period preceding the subsequent experiment.

Following the adaptation phase, the juvenile *C. gilberti* were randomly allocated into nine circular fiberglass tanks, each with a capacity of 1.3 m^3. These tanks were divided evenly among three stocking density levels (220 fish per tank). The water volume in each tank was adjusted to maintain three distinct stocking densities: 15, 25 and 35 Kg/m^3 of fish biomass, categorized as low stocking density (LSD), medium stocking density (MSD), and high stocking density (HSD), respectively. Fish were fed three times daily using commercial feed over a period of 120 days, with excess feed removed daily.

Throughout the experimental duration, parameters such as dissolved oxygen and temperature were monitored on a daily basis, while a consistent 12-hour light:12-hour dark photoperiod was maintained. At the culmination of the 120-day experimental period, ten fish were randomly selected from each tank, totaling 30 fish from each experimental group. These fish were humanely euthanized in accordance with approved animal care protocols. At the end of the experiment, the fish had an average body weight of 368.1 ± 45.5 g (HSD), 358.3 ± 38.6 g (MSD), to 334.4 ± 43.8 g (LSD) (Alvarez et al., 2020). Tissue samples were subsequently collected for gene expression analysis, following previously established procedures.

2.2. RNA extraction and obtaining coding sequences for leptin, leptin receptor and neuropeptide Y of C. gilberti

RNA extraction from each tissue began by adding 500 μL of Trizol to 50 mg of tissue, followed by homogenization in Mini beat equipment (Biospec, USA) for 10 seconds. The homogenate was then centrifuged at 8000 x g for 10 minutes at 4 °C, and the supernatant was recovered by subsequent addition of 400 μL of chloroform. After vortexing at room temperature, the solution was centrifuged at 12,000 x g for 15 minutes at 4°C. The transparent top layer was carefully transferred and loaded onto commercial E.Z.N.A. kit columns (Omega Biotec), following the manufacturer's instructions. Subsequently, RNA quantification was performed using Epoch equipment, and integrity was assessed through agarose electrophoresis.

For reverse transcription, 500 ng of each RNA sample was reverse transcribed using High Capacity cDNA Reverse Transcription Kits (Applied Biosystems™), adhering to the manufacturer's protocol.

To acquire coding sequences for leptin, its receptor, and neuropeptide Y, heterologous primers targeting conserved regions of sequences described for these genes in species closely related to *C. gilberti* were employed. The PCR products obtained underwent sequencing by Macrogen, Korea. The resulting sequences were then deposited in GenBank under the accession codes OQ954479.1 (cgLep), OQ954480.1 (cgLepR) and OQ954481.1 (cgNPY).

2.3. Bioinformatics analysis

The partial coding sequence of leptin (cgLep), leptin receptor (cgLepR) and neuropeptide Y (cgNPY) from *C. gilberti* were obtained through a contig sequence search provided by Fundacion Chile *C. gilberti* genome project. Primers for amplifying these sequences were designed using Primer3 Input software and can be found in Supplementary Table 1. Liver cDNA templates were used to amplify cgGhrelin, whereas brain cDNA templates were used to amplify cgNPY. The resulting PCR product were subsequently purified using the E.Z.N.A.® Gel Extraction Kit from Omega Biotek. The amplicon sequences were confirmed

through sequencing by Macrogen Inc. in Seoul, Korea. Sequence homology was determined using BLAST (http://blast.ncbi.nlm.nih.gov/), and the putative protein sequence was retrieved from the Expasy portal (http://www.expasy.org/).

Sequences were translated into predicted protein sequences were aligned with various known leptin sequences with the ClustalW multiple sequences alignment online server (http://www.ebi.ac.uk/Tools/mas/clustalw2/). A phylogenetic analysis was performed with Phylogeny.fr platform (http://www.phylogeny.fr/) (Dereeper et al., 2008) using the Maximum Likelihood method Saitou (Saitou, 1988). Node robustness was assessed by the bootstrap method (N=1000 pseudo-replicates).

Bioinformatics tertiary structure models for the putative mature leptin from *C. gilberti* were analyzed using the I-TASSER (Iterative Threading Assembly Refinement) web server (https://zhanggroup.org/I-TASSER/) (Yang y Zhang, 2015). The best model was chosen, and the 3D structure of the peptide was constructed using PyMOL.

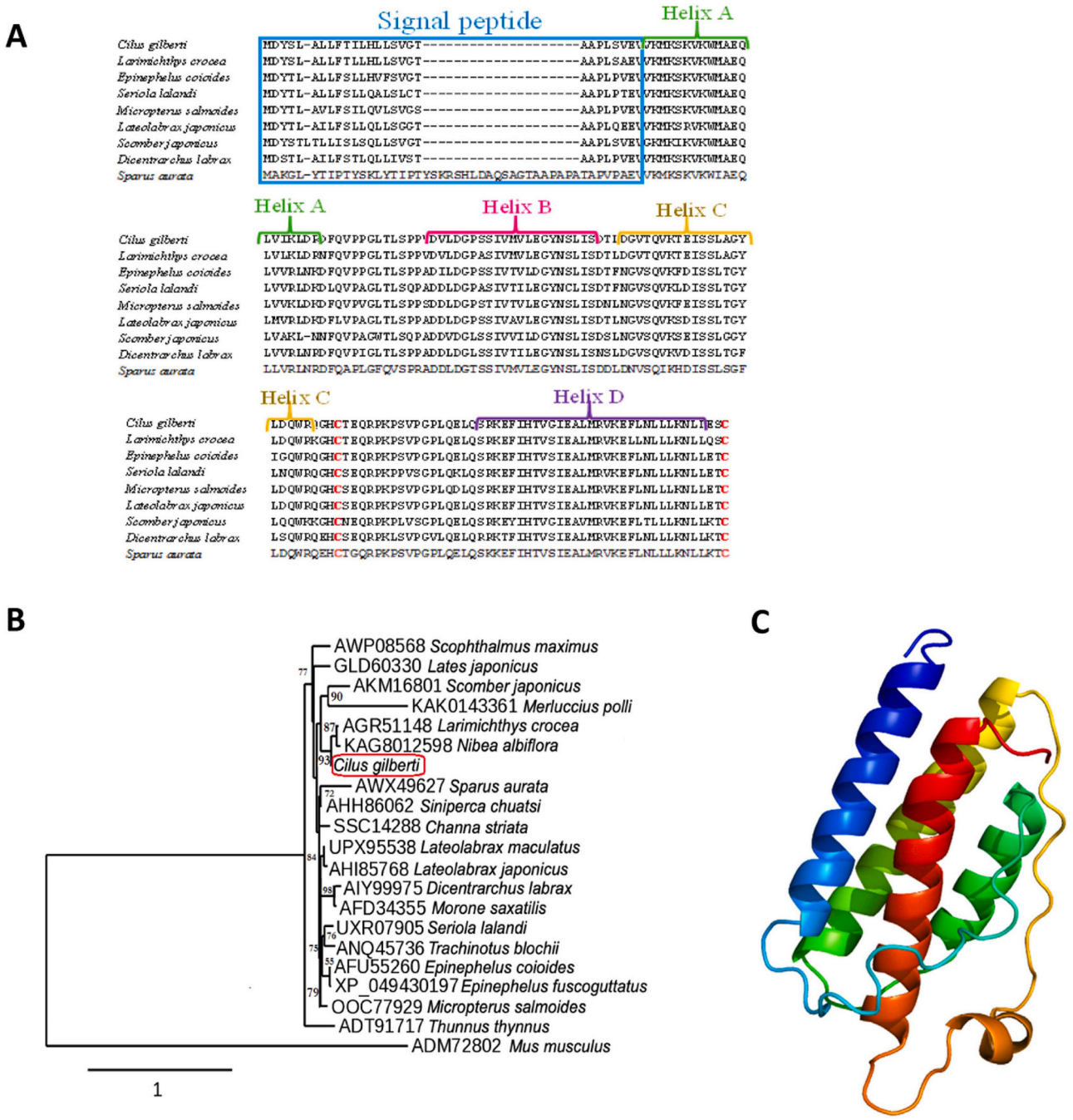

Fig. 1. Characterization of leptin coding sequence from *C. gilberti*. A) Multiple alignment of putative leptin amino acid sequences of *C. gilberti* with homologues from teleost species. Signal peptide and amino acid sequence involved in the four helix (A, B, C and D) that form the leptin structure are remarked. In addition, de two cysteine residue highly conserved between fish species is showed in red. B) Phylogenetic tree of the leptin amino acid sequence 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 leptin phylogenetic tree. C) *In silico* modeling of *C. gilberti* Leptin using the crystallographic structure of human leptin (PDB ID: d1ax8a) as a template with Helix A in blue, Helix B in green, Helix C in lemon yellow and Helix D in red.

2.4. Gene expression by RTqPCR

RT-qPCR reactions were conducted employing 10 μL reaction mixtures comprising SYBR™ Green PowerUp™ (Applied Biosystems™), 0.3 μM (final concentration) of each primer, and 2 μL of cDNA. Firstly, the efficiency of the primers (E) was determined using the equation: E = 10^(-1/slope). Assays were carried out on the QuantStudio 3 real-time systems (ThermoFisher Scientific), utilizing the following thermal profile: an initial denaturation step of 3 minutes at 95°C, succeeded by 40 cycles of PCR involving denaturation steps (95°C, 15 s), and hybridization-extension (60°C, 30 s).

Relative gene expression levels were computed employing the −2ΔΔCq method (Livak & Schmittgen, 2001). This involved using the measured quantification cycle (Cq) values of the β-actin housekeeping gene to normalize the Cq values of the gene of interest, which had been previously standardized for stock density and hypoxia assays in *Cilus gilberti* juveniles (Alvarez et al., 2020;Vega et al., 2024).

2.5. 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. The mRNA expression was subjected to one-way ANOVA followed by Tukey's mean comparisons. The blood biochemical parameters were analyzed by one-way ANOVA followed by Tukey's mean comparisons. 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.

3. Results

3.1. Characterization of the Leptin coding sequence from Chilean meagre (Cilus gilberti)

A Full-length cDNA clone was designated as CgLep (*C. gilberti* leptin) was 486 bp being the open reading frames (ORFs) complete, encoding 161 amino acids. The predicted molecular weight of the deduced protein was 17.88 kDa and the theoretical isoelectric points (pl) was 5.34. The putative protein sequence contains a signal peptide of 27 residues (MDYSLALLFTILHLLSVGTAAPLSVEV) (Fig. 1A). The ORFs exhibit the highest amino acid sequence similarity with croaker family members, such as the large yellow croaker, *Larimichthys crocea* (94.41 %) (Fig. 1A). This similarity was corroborated by phylogenetic analysis, which grouped *C. gilberti* leptin with the yellow croaker (*L. crocea*) and yellow drum (*Nibea albiflora*) in a clade, all belonging to the Family Sciaenidae. Among all the species included in the phylogenetic analysis, only one, the Atlantic bluefin tuna (*Thunnus thynnus*), was positioned outside the monophyletic clade containing the others leptin ORF (Fig. 1B).

The mature cgLep exhibits the characteristic four antiparallel α-helices previously observed in fish leptins. Notably, it contains highly conserved two cysteine residues (C111 and C161) which likely serve a crucial structural function by forming a disulfide bridge between the C and D helices (Fig. 1C).

3.2. Basal tissue expression of leptin and its receptor in C. gilberti juveniles

The analysis of basal transcriptional levels of cgLep in *C. gilberti* juvenile with an average total mass of 64.5 g revealed a markedly higher relative expression of its transcript in the liver, reaching up to 50 times higher on average compared to other tissues ($p<0.05$). This elevated expression was closely followed by the white muscle, while significantly lower levels were observed in the anterior and posterior kidney, brain, spleen, and gills ($p<0.05$). Conversely, tissues showing lower expression of cgLep include the pyloric caeca, stomach, and intestine (both anterior and posterior) (Fig. 2A). Similar results were obtained when analyzing leptin expression in tissues from larger fish subjected to normoxia conditions in the intermittent hypoxia study (average total mass of 210 g), and Chilean meagre with an average body weight of 358 g from a stock density of 25 Kg/m^3. In both cases, the highest expression was observed in the liver, followed by the muscle (Supplementary Figure 1).

Regarding its receptor, cgLepR, basal transcriptional levels appear markedly higher in the white muscle with average levels up to 300 times higher than other tissues ($p<0.05$). Then, at a much lower level of gene expression are observed in the anterior and posterior kidney, as well as the liver. Conversely, the lowest expression levels are observed in the pyloric ceca, stomach, and intestine (both anterior and posterior) (Fig. 2B).

3.3. Fed state effect on Leptin and leptin receptor expression in C. gilberti juveniles

Due to the direct impact of the animals' energy status on leptin levels, this study analyzed gene expression during both preprandial and postprandial periods.

To confirm the fasting and post-feeding conditions of the fish, the concentration of blood metabolites was initially examined before and after feeding (Fig. 3A). Results indicated a significant increase in glucose and triacylglycerides (TAG) levels after 1 hour and 6 hours post-feeding, with the highest levels observed at the latter time point ($p<0.05$). Protein concentration also exhibited a notable increase, particularly 6 hours after feeding ($p<0.05$). Notably, blood glucose levels displayed the most substantial rise after 6 hours post-feeding. Subsequently, the expression of a recognized orexigenic factor, the NPY transcript (cgNPY), was analyzed. The relative expression of cgNPY in the brain significantly increases during fasting ($p<0.05$), followed by a subsequent decrease in mRNA expression during the postprandial period (Fig. 3B).

After confirming the tissue samples were obtained during pre- and postprandial periods, the relative expression of hepatic cgLep and its receptor cgLepR at both brain and stomach levels was assessed. The results indicated low cgLep values during the preprandial period, with a significant increase observed after 1 h postprandial, followed by a decrease at the 6-hour postprandial (Fig. 3C) ($p<0.05$). Conversely, cgLepR at the brain level exhibited a significant increase during the preprandial period, with notable and sustained decreases observed at 1 and 6 hours postprandial condition. However, no changes in the gene expression level of cgLepR were observed at the stomach level when comparing preprandial and postprandial states. Additionally, expression levels were consistently lower than those detected in the brain ($p<0.05$) (Fig. 3D).

3.4. Effect of acute and intermittent hypoxia on Leptin expression in C. gilberti juveniles

Various environmental factors exert notable influences on the physiology of fish. Consequently, this study investigates the impact of both acute and intermittent episodes of low dissolved oxygen levels on hepatic leptin expression in juvenile *C. gilberti*.

The results demonstrate a significant up-regulation of leptin mRNA expression levels during the initial 2 hours of acute hypoxia exposure ($p<0.05$), followed by a return to baseline expression levels at 6 hours post-hypoxia (Fig. 4A). Conversely, fish subjected to a 30-day cycle of daily hypoxia did not exhibit significant disparities in hepatic leptin levels when compared to those maintained in normoxic conditions (Fig. 4B).

3.5. Effect of stock density on Leptin expression in C. gilberti juveniles

Stocking density stands as a pivotal determinant for achieving optimal productivity, while concurrently prioritizing the welfare of

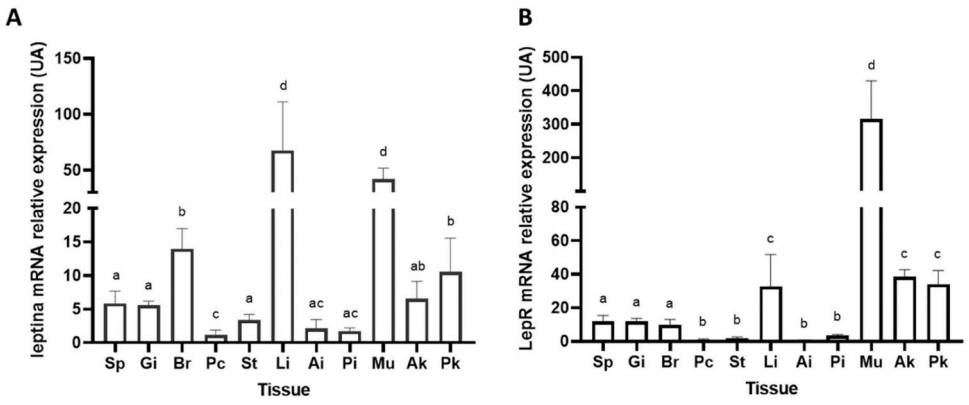

Fig. 2. Basal tissue expression of leptin and leptin receptor *from C. gilberti juveniles*. A) Leptin mRNA expression in *C. gilberti* tissues of juveniles with one-year old are expressed as relative gene expression with β-actin as housekeeping gene. B) Leptin receptor mRNA expression in *C. gilberti* tissues of juveniles with one-year old 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). Sp: spleen; Gi: gills; Br: brain; Pc: pyloric caeca; St: stomach; Li: liver; Ai: anterior intestine; Pi: posterior intestine; Mu: muscle; Ak: anterior kidney; Pk: posterior kidney.

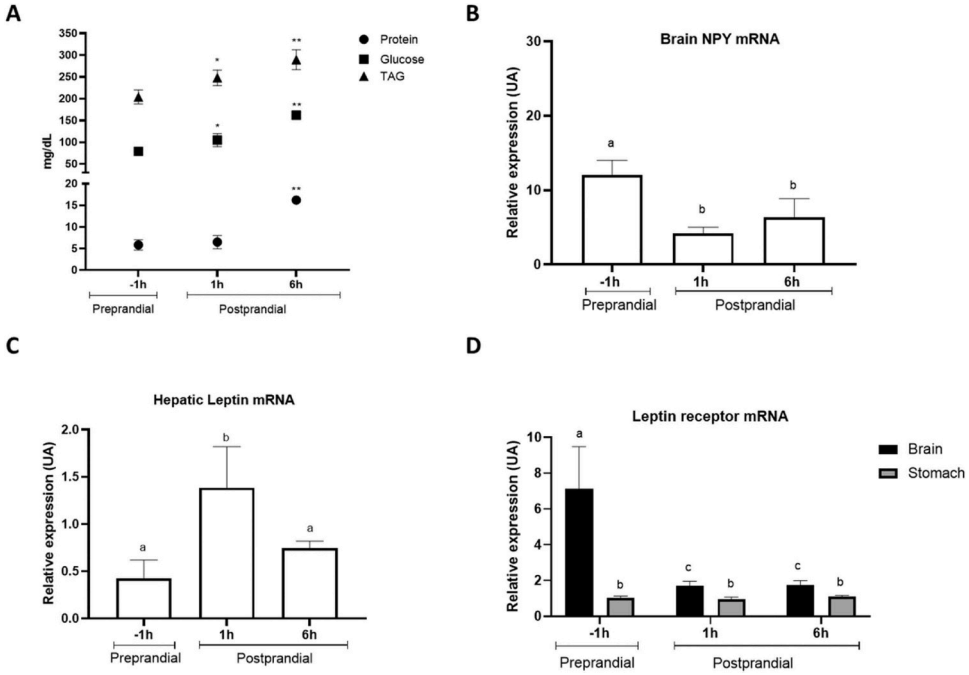

Fig. 3. Effects of prandial condition on leptin and leptin receptor expression from *C. gilberti juveniles*. A) Plasma metabolites concentration of *C. gilberti* juveniles 1 h before feeding and 1 and 6 h post feeding, B) Brain neuropeptide Y (NPY) mRNA relative expression from *C. gilbert* juveniles. C) Liver leptin mRNA relative expression from *C. gilbert* juveniles. D) Leptin receptor mRNA relative expression from *C. gilbert* juveniles. Data are given as mean ± SD and differences were considered significant when $P < 0.05$ and indicated with a different letter (n=6).

individual organisms, including the preservation of appetite signals throughout cultivation. Our conducted bioassay with Chilean meagre, reared at varying culture densities, showed interesting findings. Notably, in tanks with the lowest density (15 Kg/m^3), individual variability in hepatic leptin gene expression levels was observed. This variability manifested with some individuals exhibiting notably elevated leptin levels, while others displayed lower expression hepatic cgLep levels. Consequently, the collective average of leptin expression was notably higher in fish cultured at 15 Kg/m^3 compared to those reared at densities of 25 and 35 kg/m^3 ($p<0.05$) (Fig. 5).

4. Discussion

In this study, we undertook the comprehensive characterization of leptin's sequence and transcriptional expression in Chilean meagre (*C. gilberti*), a hormone pivotal in regulating food intake and energy balance across vertebrates (Hussain and Khan, 2017). Through the elucidation of the amino acid sequence of cgLep, we conducted an analysis of its putative tertiary structure, uncovering a composition characterized by four helices. A noteworthy observation was the conservation of two cysteine residues, which play a pivotal role in stabilizing the protein's structure. This tertiary conformation is vital as it facilitates the recognition of leptin by its cognate receptor, thus triggering the cellular signaling cascade as evidenced in vertebrates (Haglund et al., 2012;Liongue and Ward, 2007; Zabeau et al., 2005).

Analogous to mammalian leptin, these cysteine residues establish a disulfide bridge that connects the C-terminal ends of helix C and D. (Denver et al., 2011; Wen et al., 2020). However, in zebrafish lep-b, the

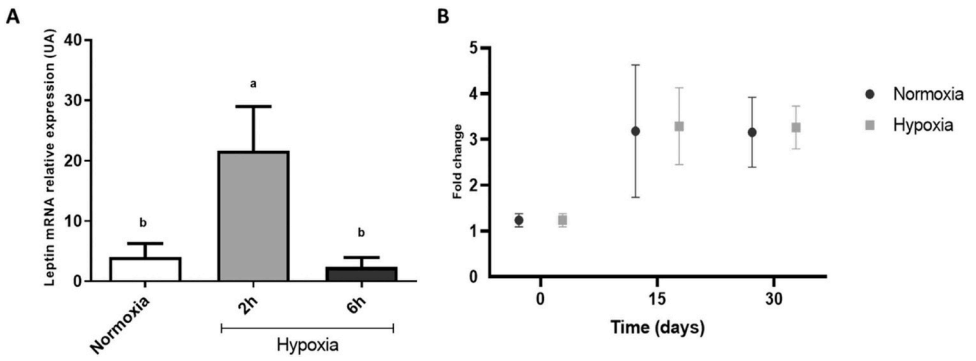

Fig. 4. Effects of acute and intermittent chronic hypoxia on hepatic leptin expression from *C. gilberti juveniles*. A) Leptin mRNA relative expression in liver from *C. gilberti* juveniles under normoxia (7,6 mg O_2/L) and after 2 h and 6 h of acute hypoxia (2 mg O_2/L). B) Leptin mRNA relative expression in liver from *C. gilberti* juveniles maintained in normoxia or daily intermittent hypoxia. Data are given as mean ± SD and differences were considered significant when $P < 0.05$ and indicated with a different letter (n=6).

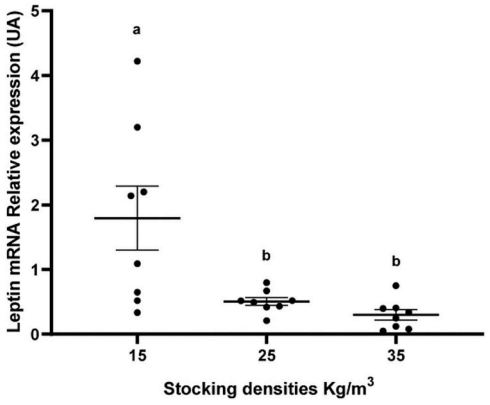

Fig. 5. Effects of stocking density on leptin expression from *C. gilberti juveniles*. Leptin mRNA relative expression in liver from *C. gilberti* juveniles stocked at 15, 25 or 35 Kg/m^3. Data are given as mean ± SD and differences were considered significant when $P < 0.05$ and indicated with a different letter (n=10).

presence of a third cysteine is demonstrated, unlike in other species such as Atlantic salmon. This distinctive feature suggests its specificity to particular species (Gorissen et al., 2009; Kurokawa and Murashita, 2009); Rønnestad et al., (2010)). As of now, it remains uncertain whether the existence of the additional cysteine could impact the hormone's functionality, potentially influencing its interaction with the receptor (Gorissen et al., 2009). In addition, the phylogenetic analysis provided support for the high degree of conservation observed in the leptin amino acid sequence among various marine fish species, as documented in previous studies conducted on teleost fish (Zhao et al., 2015; Gorissen and Flik, 2014).

While fish leptins conserve the pair of cysteine residues forming the disulfide bridge, it is of note that they lack the six residue motif GLDFIP (positions 38–43 in human leptin) completely conserved among tetrapods and required for activation of the leptin receptor (Denver et al., 2011). The absence of this motif in fish may indicate a different mechanism for binding to and activation of the leptin receptor in these vertebrates compared with tetrapods (Denver et al., 2011).

The findings of this study concerning gene expression indicate that one-year-old juveniles exhibit higher relative expression of the cgLEP transcript at the liver level, aligning with the observations made by Michel et al., (2016) in their research on zebrafish (*Danio rerio*), where both leptin and its receptor were predominantly expressed in the liver. Further support from studies on teleosts, including those by Tinoco et al., (2014); Gorissen and Flik, (2014); Trombley et al., (2012), underscores the liver's specialization in leptin hormone production.

However, unlike in mammals, leptin expression in the adipose tissue of teleost fish is minimal (Blanco and Soengas, 2021). Historically, it was believed that these fish cells did not produce the hormone (Kurokawa and Murashita, 2009). However, research by Salmerón et al., (2015) conducted in rainbow trout demonstrated that the fatty tissue of teleosts does indeed have the capacity to produce leptin, albeit at lower levels compared to higher vertebrates. Won et al., (2016) suggest that in teleosts, leptin is primarily expressed in the liver, as this tissue is responsible for energy storage in fish, including lipids, serving a function akin to mammalian fat deposits.

Notably, the expression levels of leptin in the muscle were found to be very close to those detected in the liver. Furthermore, the highest basal expression levels of cognate receptor were also observed in this tissue. These findings suggest that leptin likely plays a significant role in the muscle energy balance of *C. gilberti*. Similar patterns have been observed in studies conducted with leptin from *Oryzias latipesse*, where greater gene expression was observed at the muscle level (Kurokawa and Murashita, 2009). While the mechanism of action of leptin in lipid metabolism in fish muscle has yet to be fully elucidated, studies conducted in mice have shed light on its potential role. It has been shown that leptin is capable of promoting the oxidation of fatty acids by selectively stimulating phosphorylation and activation of the α2 catalytic subunit of AMP-activated protein kinase (AMPK) in skeletal muscle of mice (Minokoshi and Kahn, 2003). However, further research is needed to determine whether similar mechanisms are at play in fish muscle lipid metabolism of Chilean meagre.

Although the precise regulatory mechanism of leptin gene expression remains elusive, it is widely recognized as a pivotal hormone governing glucose and triacylglyceride (TAG) homeostasis (Yan et al., 2016). As elucidated by Zhang and Chua, (2018), fluctuations in nutritional status prompt variations in leptin secretion, serving as a crucial signal for the modulation of triglyceride reserves, even in non-mammalian species. Our investigation into the pre- and postprandial states in juvenile croaker reveals a dynamic relationship between hepatic leptin transcriptional levels and the availability of both TAG and glucose. Notably, two hours following ingestion, when TAG and glucose levels peak, we observed a concurrent increase in hepatic leptin expression. Given that highly active fish, such as croaker, predominantly store lipids as TAG in muscle tissue (Mcclelland et al., 1995), the heightened expression of leptin receptors in this tissue hints at a potential regulatory role in muscle fat storage. Nonetheless, further inquiry is indispensable to fully unravel the impact of leptin on lipid metabolism within *C. gilberti* muscle. These findings imply a conserved function of leptin across teleosts, primarily centered around the mobilization of energetic molecules (Chen et al., 2020). Furthermore, leptin likely exerts influence over food intake and bodyweight regulation, and adaptation to low oxygen levels in aquatic environments (Gorissen and Flik, 2014).

Feeding rhythms play a crucial role in fish metabolism, orchestrating the timing of endocrine regulators for growth and appetite. Yet, the circadian pattern of leptin, a key player in this process, remains largely unexplored in teleosts. Our research indicates a rapid surge in leptin levels post-feeding, followed by a return to near-fasting values after digestion (Huising et al., 2006). Although our focus was on transcriptional expression, studies employing recombinant leptin in teleosts suggest its ability to downregulate gluconeogenic genes such as hydroxyacyl-CoA and glucose 6-phosphatase, while simultaneously enhancing triglyceride levels through modulation of adipose triglyceride lipase and acetyl CoA carboxylase gene expression (Yuan et al., 2020). Additionally, Wu et al., (2021) reported that gibbeline carp (*Carassius gibelio*) injected with human leptin experienced heightened fatty acid oxidation, predominantly through the activation of the PI3K-AKT signaling pathway in muscle tissue. This finding supports the notion that leptin expression is triggered upon nutrient acquisition through food intake, aligning with the dynamic relationship noted between hepatic leptin transcriptional levels and TAG availability observed in *C. gilberti* and other teleosts (Gong et al., 2022).

Leptin's crucial involvement in regulating energy expenditure in response to a variety of stressors is firmly established fish often resort to reduced food intake as an adaptative mechanism when confronted with recurring stressors (Mankiewicz et al., 2021; Parker and Cheung, 2020). Among these stressors, hypoxia emerges as a critical threat, arising from inadequate oxygen saturation in aquatic environments, particularly prevalent in intensive aquaculture systems. Hence, it's reasonable to infer that hormones governing food intake might witness alterations under such environment circumstances (Conde-sieira and Soengas, 2017; Salmerón, et al., 2015). In our investigation, we employed two experimental approaches to assess whether hepatic leptin expression levels respond to low oxygen availability. Our research shown a swift rise in leptin levels within 2 hours of acute hypoxia exposure, followed by a return to normoxic levels after 6 hours. This response persists with continued hypoxia exposure over time. In our intermittent hypoxia study, fish subjected to 30 days of the same hypoxic conditions displayed leptin levels in the liver akin to those in normoxia-maintained individuals. These results suggest that this species possesses physiological adaptations that enable it to respond effectively when oxygen levels decrease in the seawater (Álvarez et al., 2020). Due to its direct association with anaerobic metabolism, hypoxia prompts organisms to rely on anaerobic glycolysis to meet increased energy demands during hypoxia-induced stress (Carmo Neves et al., 2020). Additionally, some studies propose that leptin, in tandem with glucocorticoids, may serve as a catabolic signal during stressful situations. This signal is hypothesized to facilitate the mobilization of energy resources, thereby aiding organisms in coping with the heightened energy demands brought on by stress (Parker and Cheung, 2020; Deck et al., 2017). However, in species more susceptible to hypoxia, leptin regulation may differ markedly. For instance, studies involving rainbow trout (*Oncorhynchus mykiss*) exposed to hypoxic and hyperoxic conditions demonstrated adverse impacts on weight gain, survival rates, and feed conversion, potentially linked to heightened hepatic leptin expression levels (Aksakal and Ekinci, 2021; Assan et al., 2021). Overall, appetite regulation in hypoxic environments is species-specific and depends on the life cycle of the fish.

Another significant factor that can elicit stress responses in farmed fish is stocking density, as it can impact growth, well-being, and behavior within cages. In aquaculture, ensuring fish welfare is paramount to enhancing growth rates (Zhang et al., 2022). Thus, maintaining fish at standardized culture densities often leads to higher productivity, correlated with improved food consumption—a crucial factor. In our study, we also investigated the effect of culture density on leptin transcript expression in Chilean meagre maintained at three different densities. Our findings indicate that tanks with lower fish densities exhibited higher levels of hepatic leptin expression, with average levels being elevated than densities of 25 and 35 kg/m^3. These results suggest the presence of hierarchical behavior at lower crop densities (Carbonara et al., 2019). Indeed, in tanks with lower density, two distinct groups of fish were observed: one exhibiting a high level of hepatic leptin and the other displaying a low level of hepatic leptin. This dichotomy resulted in a broad dispersion of gene expression data from fish within these tanks. The stress responses may vary among individuals, and physiological reactions might be linked to distinct environmental conditions (Øverli et al., 2007). Similar findings were reported in our previous studies (Álvarez et al., 2020), where Chilean meagre juveniles tolerate high population densities (35 kg/m^3) without experiencing chronic stress and exhibit a favorable response to acute hypoxia, without compromising growth. Absolutely, it is necessary to recognize that these behaviors are indeed species-specific. For instance, in *Salmo salar*, a contrasting effect is observed, where high population densities (greater than 20 kg/m^3) trigger the synthesis of molecules associated with appetite inhibition, such as leptin (Álvarez et al., 2022). This phenomenon could be attributed to the regulation of intake inhibition signals within the central nervous system.

Several studies indicate that leptin plays a role in modulating neuroendocrine processes related to adaptive responses and food intake behavior (Copeland et al., 2011; Gorissen and Flik, 2014). In our study, we also examined the transcriptional expression of the neuropeptide Y (NPY), known as one of the most potent orexigenic factors within the central nervous system of various fish species (Volkoff et al., 2005). Interestingly, we observed a negative correlation between hepatic leptin levels and brain NPY levels in *C. gilberti* juveniles, indicating that elevated leptin levels may reduce the expression of orexigenic neuropeptides. While these results should be validated at the protein level, findings from other teleosts injected with recombinant leptin via intracerebroventricular (ICV) or intraperitoneal (IP) routes suggest a decrease in NPY levels in species such as goldfish, nile tilapia, grass carp, mandarin fish, and rainbow trout (Blanco and Soengas, 2021; Hou and Wen, 2021; Yuan et al., 2020).

5. Conclusion

The analysis of the amino acid sequence of leptin in *C. gilberti* (cgLep) has revealed a high degree of conservation in both its sequence and structure when compared to homologs from other teleost fish species. Furthermore, cgLep is predominantly expressed in the liver and muscle tissues of juvenile Chilean meagre.

Moreover, it has been observed that cgLep gene expression increases in response to postprandial energy balance and in environments with stocking densities below 25 kg/m^3. This latter finding is particularly noteworthy, as it suggests potential alterations in feeding behavior due to heightened satiety signals resulting from changes in fish behavior.

Additionally, analyses of cgLep expression under hypoxic conditions demonstrate the remarkable resilience of this species to environments with low oxygen availability. Specifically, the upregulation of hepatic cgLep occurs only during acute hypoxia episodes, swiftly returning to baseline levels when normoxic conditions are restored.

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CRediT authorship contribution statement

Elisa Torres: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Fanny Guzman:** Writing – original draft, Supervision, Methodology, Investigation, Formal analysis. **Paula A. Santana:** Writing – review & editing, Writing – original draft,

Software, Methodology, Investigation, Formal analysis, Data curation. **Felix Acosta:** Writing – original draft, Supervision, Investigation, Formal analysis, Data curation. **Luis Mercado:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis. **CLAUDIO Andres ÁLVAREZ:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Juan F Alvarado:** Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Methodology, Investigation, Funding acquisition, Conceptualization. **Belinda Vega:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **CLAUDIA B Carcamo:** Writing – review & editing, Writing – original draft, Software, Project administration, Methodology, Data curation, Conceptualization. **Marcia Oliva:** Writing – original draft, Validation, Supervision, Resources, Investigation.

Declaration of Competing Interest

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.

Data Availability

Data will be made available on request.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.aqrep.2024.102273.

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