Aquaculture 525 (2020) 735316

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Growth performance, physiological responses to hypoxia and flesh quality of Chilean croaker (*Cilus gilberti*) stocked at different densities

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ARTICLE INFO

Keywords:
Aquaculture diversification
Stocking density
Hypoxia
Flesh quality
Marine fish

ABSTRACT

The croaker *Cilus gilberti* is an important coastal food fish in Chile and Peru, and is being considered as a target marine species for diversification of Chilean aquaculture. In order to optimize culture, responses to density and potential environmental rearing conditions must be examined. Stocking density (SD) is an important factor for cultivation-related economic viability, because inappropriate SD is frequently associated with an increase of size heterogeneity, modification of social interactions and reduction of flesh quality. In addition, drastic decreases in dissolved oxygen are one of the major recurrent environmental stress factors in the area of choice for *C. gilberti* farming in the north of Chile, thus studies on responses to hypoxia and their associations with SD are required. Therefore, in this study, *C. gilberti* juveniles prior to stocking into grow-out pens were maintained for 120 days at three different stocking densities: 15 kg/m^3 (LSD), 25 kg/m^3 (MSD) and 35 kg/m^3 (HSD), and then exposed to an acute hypoxia event. Biometric parameters, flesh quality, blood and liver biochemical parameters, and molecular markers of growth performance and stress responses were analyzed. Surprisingly, the highest body weight gain occurred in fish held at the highest density. In terms of growth-related genes, levels of growth hormone mRNA were not different between stocking densities, but liver insulin-like growth factor 1 (*IGF-1*) mRNA levels increased with SD and were the highest in the HSD group. There were no differences in flesh proximate composition. Similarly, glucose, lactate and HSP70 levels measured to assess chronic stress were not different among densities. There was a notable increase in lactate levels of fish in the HSD group in response to acute hypoxia, which suggests a greater reliance on anaerobic glycolysis by this group. In conclusion, results suggest that *C. gilberti* juveniles tolerate high stocking densities (at least 35 kg/m^3) without generating chronic stress or decreasing their capacity to respond to acute hypoxia; and high SD would be preferable due to better growth. Although present results are promising, further research is needed to continue with the standardization of the SD that will allow the best productive yield of this new fish species for Chilean aquaculture.

1. Introduction

Fish aquaculture diversification is a main productive goal worldwide because allows accessing new markets and provides resilience to climate change (Food and Agriculture Organization of the United Nations Statistical Database, 2016). Currently, the croaker *Cilus gilberti* is considered as a target marine species for the diversification of fish aquaculture by the Chilean government.

Croakers or drums belong to a group of marine fish of the Sciaenidae family, which includes about 66 genera and 291 species distributed in the Atlantic, Indian and Pacific oceans (Lo et al., 2015). Among them, some 18 species are under domestication because of the widely-regarded flavor of their white flesh (Cárdenas, 2012). *C. gilberti* (Abbot, 1899), a croaker known in Chile as corvina, is distributed along the coasts of Peru and Chile from latitudes of 6° S to 43° S, including the Galapagos Islands; and mainly associated with the soft bottom areas of

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https://doi.org/10.1016/j.aquaculture.2020.735316
Received 28 November 2019; Received in revised form 27 March 2020; Accepted 1 April 2020
Available online 03 April 2020
0044-8486/

the continental shelf (Chao and Robertson, 2015; Chirichigno, 1974; Oyarzún et al., 1999). In order to develop the culture of this species, it is necessary to establish the best conditions for development in captivity, which is in turn possible through the knowledge of its physiological responses to culture conditions.

In fish domestication, acclimation processes to different cultivation conditions are crucial for the optimal development of the animal. Fish maintained in ideal conditions develop efficiently, respond better to pathogens and stressors and display superior growth rates (Bernier and Peter, 2001; Tort, 2011). One of the principal variables to consider in fish farming is stocking density (SD), which is also an important factor for cultivation-related economic viability. Thus, optimum SD for *C. gilberti* farming is necessary in terms of maintaining a positive correlation between density and growth rate. However, this parameter must be properly evaluated to prevent chronic stress in cultivated individuals (Costa et al., 2017; Ellis et al., 2002; Iliyasu et al., 2016; Rodrigues et al., 2016). It has been reported that animal wellness decreases with an increase in density (Martins et al., 2012; Mazur and Iwama, 1993; Rotllant and Tort, 1997; Yarahmadi et al., 2016). However, low densities may also cause chronic stress (Turnbull et al., 2005).

The effect of SD on physiological and growth performance in teleost fishes depends on many factors, such as fish species, age, and rearing conditions (Refaey et al., 2018). For this reason, the effects of SD are likely species-specific responses and need to be evaluated before recommendations can be made. Inappropriate stocking density is frequently associated with an increase of size heterogeneity related to intraspecific competition for food and the modification of social interactions (Alanara and Brannas, 1996; Manley et al., 2014; Salas-Leiton et al., 2008). Furthermore, reduction of flesh quality related to the change of muscular structure has been reported (Refaey et al., 2018). Therefore, an unsuitable SD may induce great economic damage to the fish farming industry.

In addition to density, environmental culture conditions are extremely important to optimal growth. Low dissolved oxygen is one of the major recurrent environmental stress factors and a source of frequent fishery management problems (Wang et al., 2017). For *C. gilberti* culture, grow-out facilities are being established in the extreme north of Chile (Arica/Parinacota and Tarapacá regions) to take advantage of higher sea temperatures compared to central and southern Chile. However, these areas are exposed to frequent variations in dissolved oxygen (DO) due to the effects of El Niño oceanographic oscillation, upwelling of water masses with low DO, and proximity to the minimum oxygen zone (Fuenzalida et al., 2009; Helly and Levin, 2004; Paulmier and Ruiz-Pino, 2009; Sellanes et al., 2010). Therefore, studies analyzing the potential effect of SD on further tolerance to an environmental stressor such as hypoxia need to be considered for *C. gilberti* culture as well.

With the development of molecular biology technology, gene expression has become an attractive tool to evaluate physiological responses of marine economic species, such as the croakers. Transcriptomic analysis has been carried out in large yellow croaker (*Larimichthys crocea*), Mediterranean meager (*Argyrosomus regius*) and red drum (*Sciaenops ocellatus*), in order to identify molecular markers for evaluation of appropriate aquaculture conditions (González-Félix et al., 2018; Mohammed-Geba et al., 2017; Sun et al., 2017). In this way, growth hormone (GH), as well as the hepatic insulin-like growth factor type 1 (IGF-1) are two major molecular targets in the endocrine regulation of growth in teleost fishes (Côté et al., 2007; Picha et al., 2008). As in all vertebrates, somatic growth in teleost fishes has been shown to be regulated by GH in the pituitary gland. GH directly induces the production of IGF-1 in the liver which carries out most of the interaction with growing tissues. Therefore these molecules are interesting gene markers for growth rate variability studies. In fact, fish teleost GH-IGF-1 expression has been used in the evaluation of optimal temperature for somatic growth (Hevrøy et al., 2015; Levy et al., 2011), acclimation to different rearing salinities (Mohammed-Geba et al., 2017) and stocking density (Long et al., 2019; Ren et al., 2018). For physiological responses to stressors, the expression of heat shock proteins (HSPs) has been used as molecular indicators, accompanied by plasma biochemical parameters such as glucose and lactate (Mohanty et al., 2018). HSPs are an evolutionary well-conserved family of proteins, which perform essential biological roles as molecular chaperones, facilitating the synthesis and folding of proteins. It has been shown that HSP gene and protein expression can be helpful for evaluating the health status and welfare of fish in aquaculture. HSP concentrations resulting from stress induced by temperature, oxygen concentration and stocking density have been evaluated in many fish species (Antonopoulou et al., 2013; Iwama et al., 1998; Kim et al., 2018; Mohindra et al., 2015; Yarahmadi et al., 2016).

To date, there are no studies reporting the effects of farming conditions on *C. gilberti* physiology. Therefore, the aim of this study was to investigate the physiological response of Chilean croaker reared at three different stocking densities (expressed as final body weight/m^3). For this, *GH* and *IGF-1* mRNA expression was used as molecular indicators of growth performance of *C. gilberti* juveniles prior to stocking into grow-out pens, together with biometric measurements and flesh quality measurement. Moreover, potential chronic stress derived from the different SD was assessed using both plasma and liver biochemical parameters, and HSP70 protein expression. Finally, the effect the SD on the tolerance to acute hypoxia was investigated using plasma and liver biochemical parameters.

2. Materials and methods

2.1. Fish maintenance

Juveniles of *Cilus gilberti* (n = 2000; 120–130 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 circular fiberglass tanks of 1.3 m^3 with oxygen pumps, biological filters, and ultraviolet sterilizing units and flow-through seawater. Fish were fed to satiation thrice daily (09:00; 14:00 and 17:30 h) by hand from the beginning of January until the beginning of February 2017, which was considered as the adaptation period before the experiment.

After the adaptation period, juveniles of *C. gilberti* were randomly distributed into nine circular fiberglass tanks of 1.3 m^3 (three tanks at each stocking density level, 220 fish per tank) and the water volume in each tank was adjusted for to obtain and maintain three stocking densities: 15, 25, and 35 kg/m^3 biomass fish per tank. These densities were referred to as low stocking density (LSD), medium stocking density (MSD), and high stocking density (HSD), respectively. Fish were fed thrice daily (09:00; 14:00 and 17:30 h) for 120 days using a commercial feed (EWOS Chile – Cargill, Puerto Montt, Chile). Excess feed was siphoned daily from the tanks.

During the experimental period, dissolved oxygen (DO) (7.4 mg/mL ± 0.7) and temperature (18.1 °C ± 2.2) were tested daily using a multi parameter probe (YSI, Inc.). Fish were held under a 12 L:12D photoperiod. No variations in water quality parameters were detected among experimental groups. Neither chemical nor antibiotic treatments were used at any time.

Ten fish from each tank (30 from each experimental group) were randomly collected from the main SD experiment at the end of the experimental period (120 days) to evaluate several physiological parameters, including blood biochemical parameter, proximate composition and the expression of molecular markers. Sampled fish were captured and quickly anesthetized with a sub-lethal dose of AQUI-S® (13.3 mg/L, Isoeugenol 50%, Bayer-Germany). Blood samples were immediately taken from the caudal vein using heparinized syringes. After blood collection, the fish were sacrificed by decapitation (according to the institutional approved animal care protocols), and tissue samples (liver and pituitary gland) were obtained for gene expression analysis.

Moreover, a section of muscle was obtained for proximate composition analysis. Biometric parameters of each animal were recorded.

2.2. Acute hypoxia stress

A second group of fish from the main SD experiment was used for the hypoxia response analyses. After 120 days at different stocking densities, six fish of each tank (i.e., $n = 18$ per SD) were randomly transferred at the same time into two tanks (100*L*), with 3 fish in each tank. One tank was maintained at normoxia (7.5 ± 0.5 mg/L) and the other subjected to hypoxia (2.0 ± 0.1 mg/L) for 2 h through injection of nitrogen gas into the water. Dissolved oxygen (DO) and temperature were monitored every fifteen minutes during the acute hypoxia exposure using ProDSS (digital sampling system) handheld multiparameter meter (YSI Inc. / Xylem Inc). Following normoxia or hypoxia exposure, fish were anesthetized with 13.3 mg/L AQUI-S® (isoeugenol 50%, Bayer-Germany). Fish were sampled for liver, muscle and blood. The blood samples were centrifuged immediately at 3000 rpm for 5 min at 4 °C, and then serum was removed and stored at −80 °C until use. The liver and muscle specimens were frozen in liquid nitrogen and stored at −80 °C soon after sampling. This procedure was repeated for each tank.

2.3. Biometric analysis

Total length (TL) was measured to the nearest millimeter from the tip of the snout to the end of the middle caudal fin rays. The total weight (TW) was determined as the total body weight with a digital balance to an accuracy of 0.01 g. Fulton's condition factor (K), was calculated using the equation: $K = (TW/TL^3)*100$ (Froese, 2006). Growth was calculated as specific growth rate (SGR) according to the equation: SGR = (lnTW2-lnTW1)*100/(T2-T1); were TW1 and TW2 are total weight at days T1 (start of experiment) and T2 (after 120 days).

2.4. Proximate composition

Proximate composition of *C. gilberti* muscle was determined in triplicate (six fish pooled in each tank). Crude protein content was determined by the Kjeldahl method (AOAC 2000). Total lipids were evaluated after chloroform-methanol (2:1, *v*/v) lipid extraction (Folch et al., 1957). Fatty acid methyl esters (FAME) of muscle were prepared according to Christie, (1989) by transesterification with 1% sulphuric acid in methanol. FAME were purified using Sep-pack NH_2 filters (Waters SA, Milford, Massachusetts) as described by Fox, (1990) and analyzed in a gas chromatograph (Shimadzu GC-14, Kyoto, Japan) with a flame ionization detector and a capillary column (Omega-wax 320, Supelco/Sigma-Aldrich; 30 m × 0.32 mm, film thickness 0.25 mm).

2.5. Biochemical assays

Glucose, triglycerides (TAG) and lactate levels from plasma were measured using commercial kits from Spinreact (Spain) following the manufacture protocols. Liver glycogen concentration was quantified using the method from Keppler and Decker (1974) (Keppler and Decker, 1974). Glucose after glycogen breakdown with amyloglucosidase (Sigma-Aldrich A7420) was measured with a Spinreact commercial kit (Spain).

2.6. Total RNA extraction and gene expression analysis by qPCR

Total RNA of *C. gilberti* tissue was extracted from tissues using AxyPrep Multisource Total RNA Miniprep Kit (Axygen Biosciences, Union City, CA, USA). RNA was then treated with DNAse I (Promega), 15 min at room temperature and inactivated by heat, 10 min at 65 °C. Next, total RNA was quantified with an Epoch spectrophotometer (BioTek, Winooski, VT, USA) and the quality was determined by agarose gel electrophoresis. cDNA synthesis was carried out using PrimeScript™ RT Reagent Kit with gDNA Eraser (TaKaRa, Japan) and oligo-$p(dT)_{15}$ primer following the manufacturer protocol.

Specific primers were designed to amplify growth-associated genes for *C. gilberti*: growth hormone, *CgGH* (GenBank accession no. submitted 2,305,756) (Forward 5′ TCAGGACGCAGCAGAAATCTTT 3′ and reverse 5′ GTCTTTCTTGAAACAGGCGAGC 3′); insulin-like growth factor type 1, *CgIGF-1* (GenBank accession no. submitted 2,305,756) (Forward 5′ ACAAAGTGGACAAGGGCACA 3′ and reverse 5′ CAAGTC GCTGGGCATTTGTCCATTC 3′); and the endogen gene *Cgβ-actin* (GenBank accession no. submitted 2,305,756) (Forward 5′ TATCGTCA TGGACTCCGGTGAT 3′ and reverse 5′ CTTGATGTCACGCACGATTTCC 3′). In order to validate *β-actin* as a housekeeping gene for our samples, statistical tests on *β-actin* expression values among different tissues or stress conditions were performed, and non-significant differences were found among them ($P > .05$). RT-qPCR was performed using 20 μL reaction mixtures containing Maxima® SYBR Green/ROX qPCR Master Mix (Thermo Scientific, Rockford, IL, 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: $E = 10^{[-1/\text{slope}]}$ from 6 serial dilutions of pooled cDNA for each primer pair. Assays were carried out in a Mx3000P qPCR System (Agilent Technologies) with an initial denaturation step of 10 min at 95 °C followed by 40 PCR cycles of 95 °C, 15 s and 60 °C, 1 min and finally the melting curve (95 °C/15 s, 55 °C/15 s, and 95 °C/15 s). Relative expression was calculated using the $-2\,\Delta\Delta$Cq 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 genes.

2.7. HSP70 protein quantification by ELISA

Total proteins in liver tissue samples were determined using bicinchoninic acid assay (Pierce BCA protein assay kit, Thermo Scientific, USA) using bovine serum albumin as the standard. Commercial anti-Hsp70 antibody (3A3, Abcam 5439) was used to quantify HSP70 protein levels. Firstly, antibody specificity in both liver and blood samples was determined by Western blot (Supplementary Fig. 1). For *C. gilberti* liver tissue, only one band at the level of 70 kD was observed, which is indicative of the detection of one HSP70 isoform. The HSP70 protein level was quantified by indirect enzyme-linked immunosorbent assay (ELISA). Total protein of liver samples was diluted in 0.05 M carbonate-bicarbonate buffer (pH 9.6), and 50 μL of the sample (40 μg of total protein) was aliquoted per well and incubated in an ELISA plate overnight at 4 °C with three blanks (buffer only). The plate was then washed with phosphate-buffered saline (PBS) and 200 μL of blocking buffer (PBS + 5% skim milk) was subsequently added to each well and incubated for 2 h. The wells were washed again with PBS. Subsequently, 100 μL (40 ng/μL) of the primary antibody (Anti-Hsp70 antibody) diluted in blocking buffer + 0.05% Tween-20 was added to each well and incubated overnight at 4 °C. The plate was then washed with PBS and then incubated with a goat anti-mouse IgG-HRP secondary polyclonal antibody (Thermo Fisher Scientific), diluted at 1:2500 in blocking buffer + 0.05% Tween-20 for 2 h at 25 °C, and washed again four times with PBS. Then, 100 μL per well of 3,3′,5,5′-Tetramethylbenzidine (TMB) single solution (Invitrogen) was added and incubated for 30 min at room temperature; the reaction was stopped with 50 μL of sulphuric acid 1 N and read at 450 nm with a microplate spectrophotometer. The absorbance of the sample was corrected by the mean absorbance of the blanks and divided by a conversion factor, which was estimated from a linear regression curve of cognate HSP70.

2.8. Statistical analysis

In order to determine statistical differences among treatments, one-way analyses of variances (one-way ANOVAs) were used to compare

growth and chronic stress responses. Two-way ANOVAs were used to compare responses to acute hypoxia, with SD and oxygen levels as the main factors. Tukey post-hoc test for data were analyzed using R version 3.5.2 software. Differences were considered significant when $P < .01$ (**) or $P < .05$ (*). Results are represented graphically as the mean ± standard deviation of the biological replicates.

2.9. Ethics statement

Fish were kept and handled following the guidelines of experimental procedures in animal research from the Ethics and Animal Welfare of National Commission for Scientific and Technological Research (CONICYT) and following the Chilean legislation.

3. Results

3.1. Growth performance

At the end of the experiment, which happened 120 days after transference of *C. gilberti* juveniles to the three different stocking densities (SD), a clear increase in weight was observed in all groups tested. However, weight gain (starting at 147.7 ± 2.0 g) of specimens held at the highest stocking density (35 kg/m^3 or HSD group) were greater than the lowest stocking density group (LSD) (Fig. 1) with mean final body weight of 368.1 ± 45.5 g and 334.4 ± 43.8 g, respectively. Besides, gains in total length (starting at 22.7 ± 0.1 cm) did not differ among SD (Fig. 1); and neither did the condition factor (K) and specific growth rate (SGR), which varied between 0.67 ± 0.11–0.75 ± 0.10 and 1.25 ± 0.15–1.30 ± 0.07, respectively.

3.2. Expression of genes related to the growth

The transcriptional expressions of genes related to growth of *C. gilberti* (GH/IGF-1 axis) were evaluated by RT-qPCR. The levels of *GH* mRNA in pituitary did not show significant differences among the different stocking densities (Fig. 2A). In contrast, liver *IGF-1* mRNA levels increased with SD, and were the lowest in the LSD group and the highest in the HSD group (Fig. 2B).

3.3. Flesh proximate composition

Proximate analyses showed that *C. gilberti* muscle contained high crude protein (mean of ≈65.7% dry weight) and moderate lipid levels (mean of ≈ 20.7% dry weight) (Table 1). Crude protein and total lipid levels were not statistically different among stocking densities tested. Interestingly, there was a wide variation in crude protein and total lipids level in both the MSD and HSD groups. Muscle moisture and ash percentages were significantly higher in the LSD than in the HSD group (Table 1).

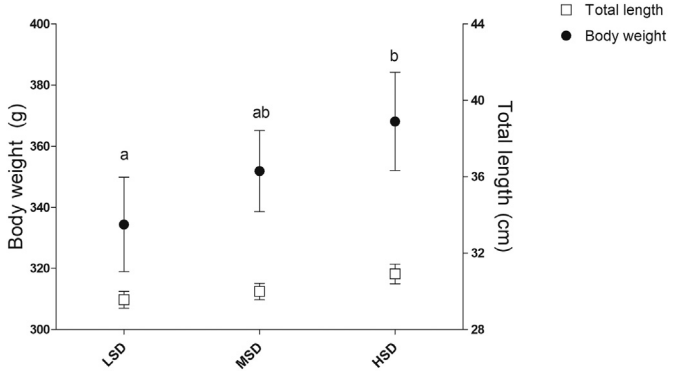

Fig. 1. Effect of stocking density on *Cilus gilberti* juvenile's growth performance. Values of body weight and total length of juveniles cultured at different initial stocking densities for 120 days are expressed as mean ± SD ($n = 30$). LSD: low stocking density (15 kg/m^3); MSD: medium stocking density (25 kg/m^3); HSD: high stocking density (35 kg/m^3). Different letters indicate significant differences between experimental groups ($P < .05$, one-way ANOVA followed by Tukey's test).

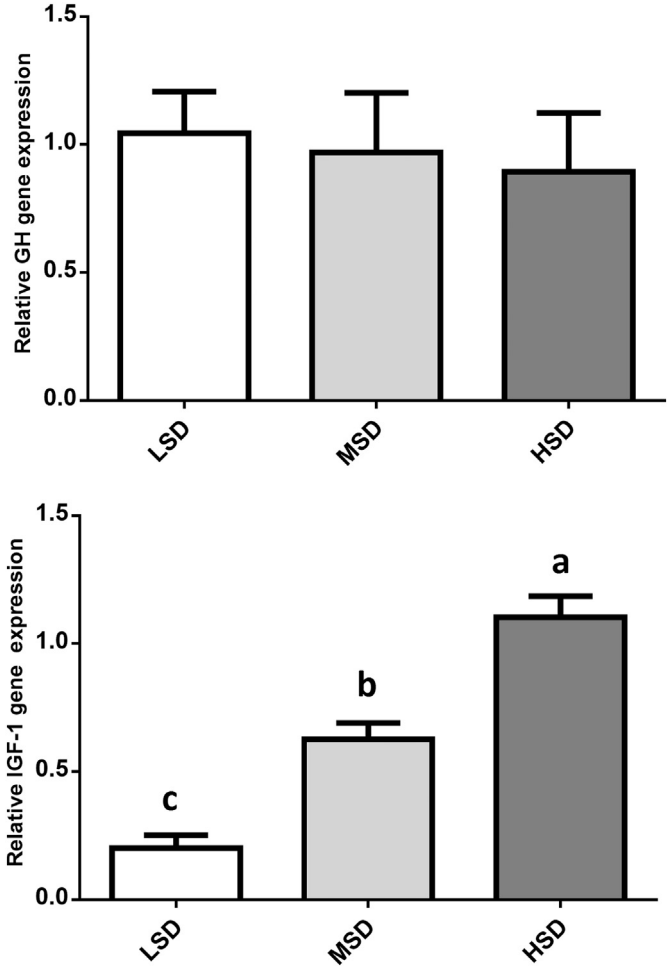

Fig. 2. Relative expression of growth-related genes in juveniles of *Cilus gilberti* cultured at different initial stocking densities for 120 days. Effect of stocking density on pituitary growth hormone *CgGH* (A) and hepatic *IGF-1* (B) mRNA expression levels. Data are presented as mean ± SD ($n = 12$). LSD: low stocking density (15 kg/m^3); MSD: medium stocking density (25 kg/m^3); HSD: high stocking density (35 kg/m^3). Different letters indicated significant differences between experimental groups ($P < .05$, one-way ANOVA followed by Tukey's test).

Table 1
Flesh proximate composition of *Cilus gilberti* juveniles cultured at different stocking densities.

Stock density/ Proximate composition	15 kg/m^3 (LSD)	25 kg/m^3 (MSD)	35 kg/m^3 (HSD)
Crude protein (%)[1]	67.6 ± 4.0	62.9 ± 9.9	67.8 ± 1.0
Total lipids (%)[1]	13.1 ± 0.4	23.3 ± 10.7	24.2 ± 9.5
Ash (%)[1]	5.0 ± 0.1 [a]	4.3 ± 0.2 [b]	4.3 ± 0.1 [b]
Moisture (%)[2]	71.6 ± 0.4 [a]	70.1 ± 0.7 [ab]	70.3 ± 0.5 [b]

[1] The content is expressed as percentage of dry weight; [2]The content is expressed as percentage of wet weight. Values (mean ± SD) in the same row with the same superscript letter are not significantly different ($P > .05$).

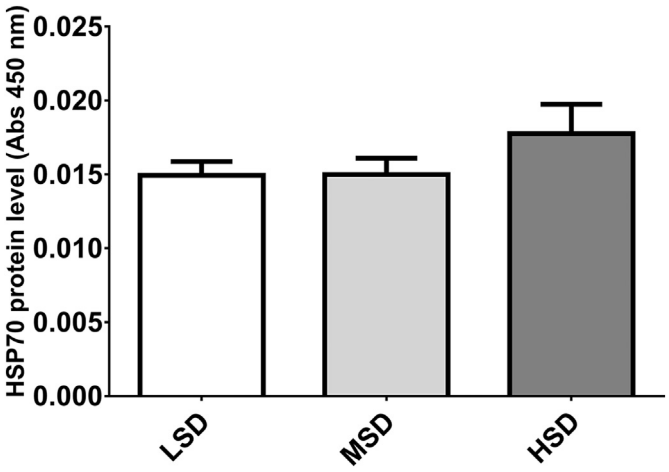

Fig. 3. Levels of HSP70 in liver of juveniles of *Cilus gilberti* cultured at different initial stocking densities for 120 days. HSP70 protein level was measurement by indirect ELISA. Data are presented as mean ± SD (n = 12). LSD: low stocking density (15 kg/m^3); MSD: medium stocking density (25 kg/m^3); HSD: high stocking density (35 kg/m^3).

3.4. Molecular and biochemical parameters associated with chronic stress response

Protein levels of HSP70 in the liver, as well as plasma glucose and TAG, and hepatic glycogen and TAG were evaluated as a chronic stress indicator. There were no differences in HSP70 levels among the three groups (LSD, MSD and HSD), although there was a trend toward higher HSP70 in the HSD group (Fig. 3). Similarly, no differences were observed in the basal levels of plasmatic or hepatic parameters among SD groups (Fig. 4, open bars).

3.5. Biochemical parameters associated with response to acute hypoxia

The interaction between stocking density and oxygen level affected significantly plasma glucose and lactate levels (Fig. 4A-B). Following acute hypoxia, the levels of plasma glucose increased significantly in the LSD and HSD groups respect to their levels under normoxia (Fig. 4A). In addition, acute hypoxia induced significant increase in lactate concentration in the plasma (respect to their levels under normoxia) of juveniles maintained under the three SDs; but this increase was higher in the HSD group than both MSD and LSD groups (Fig. 4B). Plasmatic TAG did not differed significantly among juveniles maintained under normoxia and exposed to hypoxia in the three SDs (Fig. 4C).

There were no differences in liver metabolites (glucose, glycogen and triglycerides) following hypoxia exposure among density treatments (Fig. 4D-F).

4. Discussion

Stocking density is an important rearing condition for successful fish culture. Assessing physiological responses to stocking densities (SD) for new fish culture allows for operational improvements, which favor low-cost management systems for production. In our study, *Cilus gilberti* juveniles that were held at the highest stocking density (HSD) showed the most weight gain after 120 days of culture. This result was consistent with the molecular analysis of hepatic *IGF-1* mRNA, which revealed the highest levels in juveniles of the HSD group, in comparison with fish from the medium and low stocking densities (respectively MSD and LSD) groups. As described in previous studies, good growth performance is expected with low pituitary *GH* mRNA in combination with a high concentration of hepatic *IGF-1* (Mun et al., 2019; Pérez-Sánchez and Le Bail, 1999). IGF-1 plays a critical role in both somatic growth and muscle function in fish (Dai et al., 2015; Mun et al., 2019), therefore, the high level of hepatic *IGF-1* mRNA supports the greater growth of *C. gilberti* maintained at high stock densities.

This positive relationship between stocking density and growth observed on *C. gilberti* juveniles confirms findings reported in other croakers, such as meager (*Argyrosomus regius*), red drum (*Sciaenops ocellatus)* and Japanese meager (*A. japonicas*) (Collett et al., 2011; Millán-Cubillo et al., 2016; Pirozzi et al., 2009; Vela et al., 2019). Feed utilization is improved in fish held at high stocking densities (Millán-Cubillo et al., 2016). However, it has been demonstrated that high stocking density may suppress growth of other farmed fish species due to enhanced aggression and competition for food (Ellis et al., 2002; Martins et al., 2012). The effects of aggression associated with stocking density are still unclear, existing studies suggest that high density may either increase or reduce aggressiveness (Martins et al., 2012). Therefore, for the domestication process is relevant to considerer the social behavior and cycle stage live of the fishes species being cultured to define an appropriate stocking density (Karakatsouli et al., 2007).

Croakers are well known for their ability to make sound (Ladich, 2019), a special adaptation for social communication. Shoal formation or cohesive group behavior is important in social fish species, and can reduce stress responses (Allen et al., 2009). The primary function of schooling behavior in fish is predator avoidance (Pavlov and Kasumyan, 2000). In addition, evidence for involvement of shoaling in feeding, growth and migratory behavior have also been described (Pavlov and Kasumyan, 2000; Peuhkuri et al., 1995; Stirling, 1977). Shoaling behavior and feeding responses have been described during culture of *A. regius* at higher densities (Millán-Cubillo et al., 2016). This information suggests that a lower threshold of stocking density may also affect the social cohesiveness of *C. gilberti* in rearing conditions associated with aquaculture. It is possible that improved growth observed at the highest density and decreased growth at lower densities relates to social adaptations and feeding facilitation in this species. Therefore, further research focusing on both feeding behavior and regulation of *C. gilberti* juveniles may be beneficial for the domestication process of this species.

In the present study, no differences in crude protein and total lipids levels associated with the stocking densities tested were found. Fillet constituents were similar to other farmed sciaenid fishes species such as *A. regius* and *Sciaena umbra* (Cakli et al., 2006; Grigorakis, 2017; Poli et al., 2003). Nevertheless, in our study we also observed a wide variation in lipid content of *C. gilberti* flesh in both the MSD and HSD groups. In previous studies, it has been described that high stocking densities have a more profound effect on fat mobilization as a result of respective metabolic adaptations to help meet the increased energy demand (Montero et al., 1999). In addition, these differences can be attributed to feed intake variances (Grigorakis, 2017), confirming the need for further feeding behavior studies.

Increasing density to ensure optimal growth performance of *C. gilberti* must be evaluated to prevent consequences on several physiological processes such as activation of stress pathways and associated wellness reduction. In addition, it has been described that many stressors induced a flesh quality loss (Refaey et al., 2017). Factors that cause prolonged stress can drastically alter the energy balance of fishes, increasing plasma metabolites such as glucose and decreasing liver glycogen, a common secondary response associated with extra energy expenditure to prepare an animal for an escape “fight or flight” response, or to adapt to or reduce the impact of a stressor (Wendelaar Bonga, 1997). Overall, our results demonstrate that baseline plasma metabolites were not different among the densities tested. Another option to evaluate activation of the stress response is through the molecules involved in stress response. Previous studies indicate that HSP70 measurement could be used as stress biomarker for fish (Celi et al., 2012). In fact, increasing stocking density induces the expression of stress related protein HSP70 in rainbow trout and tilapia (Aksakal et al.,

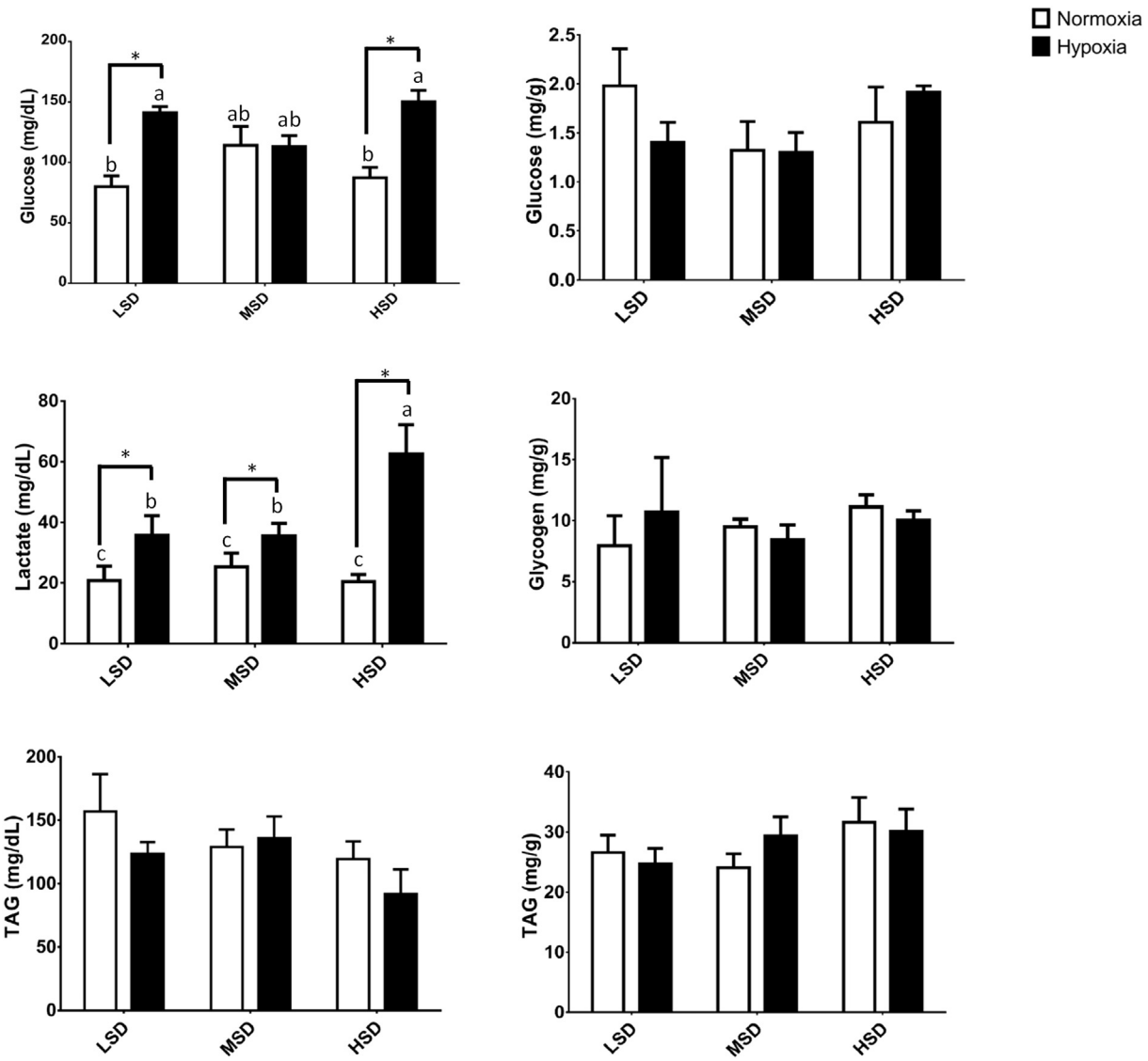

Fig. 4. Effect of stocking density and acute hypoxia on liver and plasma biochemical parameters in *Cilus gilberti* juveniles. Values of glucose (A), lactate (B) and TAG (C) plasma biochemical parameters; and glucose (D), glycogen (E) and TAG (F) liver biochemical parameters measured after cultured at different stocking densities for 120 days and then exposed to acute hypoxia. Bars represent mean ± SD (n = 30). LSD: low stocking density (15 kg/m^3); MSD: medium stocking density (25 kg/m^3); HSD: high stocking density (35 kg/m^3). Significant differences among stocking densities and/or between normoxia and hypoxia experimental groups are indicated (respectively by asterisks or different letters; $P < .05$, two-way ANOVA followed by Tukey's test).

2011; Ridha, 2006). In the present study, hepatic HSP70 protein levels were similar in all densities of *C. gilberti* juveniles. Thus, both indicators suggest that the high assessed stocking density does not induce chronic stress in juveniles of *C. gilberti*. Although because there was a trend toward higher HSP70 in fishes in the highest stocking density, further studies are needed to elucidate how high a density will be tolerated by *C. gilberti* while maintaining growth and minimizing chronic stress.

Oxygen is essential for oxidation of metabolic fuels required to maintain basal metabolic rate and for performing various activities essential for survival. However, oxygen availability in aquatic habitats can change drastically, and be strongly reduced resulting in a hypoxic environment. In addition, the frequency of these events is increasing every year as the result of anthropogenic nutrient enrichment and climate change (Conley et al., 2011; McCormick and Levin, 2017). In this regard, the intensive aquaculture of *C. gilberti* is planned for northern Chile where periodic cycles of environmental hypoxia have been recorded. This region is exposed to frequent variations in DO due to the effects of El Niño oceanographic oscillation, upwelling of water masses with low DO, and proximity to the minimum oxygen zone (< 0.64 mg/L) (Fuenzalida et al., 2009; Helly and Levin, 2004; Paulmier and Ruiz-Pino, 2009; Sellanes et al., 2010). Thus, to evaluate the potential accumulated effects of being previously reared under different SD on future tolerance to acute hypoxia was also investigated herein. In our study, physiological effects of an acute hypoxic event (2 h at 2 mg O_2/L) increased both plasma glucose and lactate concentrations in juveniles of *C. gilberti*. Along with physiological adjustments in response to hypoxia, a biochemical change in energy metabolism takes place whereby anaerobic glycolysis becomes the major pathway for ATP production. Anaerobic metabolism is recruited in teleosts under conditions of intense exercise or lowered environmental oxygen availability, typically resulting in the accumulation of lactate in blood and tissues (Omlin et al., 2010; Richards et al., 2009; Virani and Rees, 2000). Interestingly the lactate levels of HSD group were higher than those in MSD and LSD group after hypoxia exposure. Also, HSD group presented the lowest plasmatic TAG levels after acute hypoxia exposure. Parallel decrease of TAG with increase in lactate levels have been observed after hypoxia exposure in mammals by Sun et al. (2016). These authors suggest that this would be a mechanism that promotes lipid accumulation in muscle

cells for mitochondrial maintenance under hypoxic conditions (Sun et al., 2016). Thus, our data suggest that fish in the highest density had greater reliance on anaerobic glycolysis under acute hypoxia than fish held at the lowest stocking density, leading to an accumulation of lactate when seawater dissolved oxygen decreased. This result may also hint a faster response to hypoxia of *C. gilberti* maintained under HSD. Future studies would benefit from an examination of successive hypoxic events on physiological responses of *C. gilberti* in combination with high density, for guidance of culture practices.

Overall, our results of physiological analyses represent the first published data on stock density effects in the growth performance and tolerance to acute hypoxia of *C. gilbert*. Results suggest that *C. gilberti* juveniles tolerate high densities (at least 35 kg/m^3) without generating chronic stress or decreasing their capacity to respond to acute hypoxia; and high SD would be preferable due to better growth. Although present results are promising, further research is needed to continue with the standardization of the stocking density, which will allow improving the management and providing the best productive yield of this new fish species for Chilean aquaculture.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2020.735316.

Funding

This work was supported by a grant from ANID-Chile, FONDECYT 11180042 to C.A., and Programa Integrado para el Desarrollo Sustentable del Cultivo de Corvina (*Cilus Gilberti*) (CORFO-INNOVA 09PDAC-6912) to Fundación Chile. IJC was supported by International Postgraduate Doctoral Scholarship Program from University of Cádiz (UCA/R58REC/2016).

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.

Acknowledgments

We gratefully acknowledge Dr. Peter Allen for english revision of the manuscript.

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