The influence of stocking density and food deprivation in silver catfish (*Rhamdia quelen*): a metabolic and endocrine approach

Charlene Menezes[1], Ignacio Ruiz-Jarabo[2], Juan Antonio Martos-Sitcha[2,3], Cândida Toni[4], Joseânia Salbego[4], Alexssandro Becker[4], Vania Lucia Loro[1], Gonzalo Martínez-Rodríguez[3], Juan Miguel Mancera[2], Bernardo Baldisseroto[4*]

[1] Departamento de Química, Universidade Federal de Santa Maria, Santa Maria, RS, Brasil.

[2] Departamento de Biología, Facultad de Ciencias Del Mar y Ambientales, Universidad de Cádiz, Puerto Real, Cádiz, Spain

[3] Instituto de Ciencias Marinas de Andalucía, Consejo Superior de Investigaciones Científicas, Puerto Real, Cádiz, Spain

[4] Departamento de Fisiologia e Farmacologia, Universidade Federal de Santa Maria, Santa Maria, RS, Brasil

* Corresponding Author
Departamento de Fisiologia e Farmacologia
Universidade Federal de Santa Maria
97105.900 – Santa Maria, RS, Brazil
Phone: +55-55-3220-9382, fax: 55-55-3220-8241
e-mail: bbaldisserotto@hotmail.com

Abstract

The influence of stocking density and food deprivation on energy metabolism, stress processes and pituitary endocrine system of silver catfish (*Rhamdia quelen*) was investigated after a period of 14 days, assessing plasmatic and hepatic parameters, as well as the mRNA expression of prolactin (PRL), growth hormone (GH) and somatolactin (SL). Fish were submitted to four different experimental conditions: (1) fed under high stocking density (32 kg/m^3, HSD); (2) fed under mean stocking density (16 kg/m^3, MSD); (3) fed under low stocking density (8 kg/m^3, LSD); (4) food-deprived fish under low stocking density (8 kg/m^3, LSD-FD). After 14 days, plasma and liver samples were taken for the analysis of metabolites levels and enzymatic activities related to metabolism, and pituitary glands were taken for the analysis of expression of hormones (PRL, GH and SL). Liver weight and hepato-somatic index (HSI) revealed that specimens maintained at HSD and/or MSD groups presented higher amounts of hepatic stores, as seen in triglycerides and glycogen levels in this tissue, than animals submitted to low density, with or without feeding. Triglycerides levels in plasma and liver revealed consumption of fatty acid reserves in the fasting group. Enzymatic activities like glutamate dehydrogenase (GDH), phosphorilase (GPase), pyruvate kinase (PK), aspartate transaminase (AST) and glycerol-3-phosphate dehydrogenase (G3PDH) pointed to an increase in gluconeogenic pathways in the HSD group, as well as an increase in glucolitic metabolism in the LSD groups. The expression of PRL was not affected by the stocking density and/or food deprivation and GH reduced with increased density, and increased in fasting conditions. A negative effect of density and fasting was observed on the expression of SL. Taken together, data suggested that juveniles of the silver catfish reared at densities of 16 to 32 kg/m^3 were maintained at better stocking conditions than those at the lowest density.

Keywords: fish, hepato-somatic index, metabolism, pituitary hormones

1. Introduction

Studies of stress in fish have often been made in the field of physiology (Arjona et al., 2010; Costas et al., 2011; Herrera et al., 2012; Ruane et al., 2002). In the environment, the stress response can be seen as the ability of fish to mobilize energy reserves to avoid or overcome situations of threat. On the other hand, in fish culture systems the stress situation is constantly present and may affect the productive development, harming the health of fish and increasing susceptibility to disease (Barcellos et al., 2011).

The primary stress is characterized by a significant increase of corticosteroid hormones (cortisol) and the concentration of catecholamines (epinephrine and norepinephrine) that stimulate the hydrolysis of glycogen in the liver, increasing the levels of blood glucose, decreased muscle protein and increased heart rate (Barton, 2002). These hormones induce secondary stress responses, characterized by the reduction in the hepatic glycogen content and increased plasma glucose levels in order to provide glucose to tissues for homeostasis (Barton, 2002). In a chronic stress situation, tertiary responses could affect whole-animal changes such as growth, reproductive efficiency, disease resistance and behavior (Barton, 2002; Wendelaar-Bonga, 1997).

In the aquaculture activity, stocking density is also of concern for fish culture. Previous studies have been focused on evaluating the effects of stocking density on fish growth, behavior, metabolism, physiological and biochemical parameters (Herrera et al., 2009; Li et al., 2012; Montero et al., 1999; Sangiao-Alvarellos et al., 2005b). The stocking density is a stressor that activates stress response in fish, affecting different metabolic enzymes related to lipid, carbohydrate and protein metabolism (Costas et al., 2008; Laiz-Carrión et al., 2012; Montero et al., 1999; Sangiao-Alvarellos et al., 2005a).

Food deprivation is also a common type of stress in fish that induces energy-releasing catabolic processes that compensate the reduced energy intake (Wunderink et al., 2012). At least during the initial stages of fasting, the maintenance of glycemia is directly related to the capacity of mobilization of hepatic glycogen and also depends on the subsequent activation of hepatic gluconeogenesis and the subsequent reduction of the rate of glucose utilization (Navarro and Gutiérrez, 1995).

Prolactin (PRL), growth hormone (GH) and somatolactin (SL) are considered as belonging to the same hormone family due to their structural similarities, however present different functions related to diverse physiological processes (Manzon, 2002; Pérez-Sánchez, 2000; Vega-Rubín de Celis et al., 2004). PRL is essential for acclimation to hyposmotic environments, being also related to processes such as reproduction, stress and metabolism (Laiz-Carrión et al., 2009; Mancera and McCormick, 2007). In addition, GH regulates growth, intermediary metabolism and in some species has osmoregulatory effects (Mancera and McCormick, 2007; Sakamoto and McCormick, 2006). SL is related to different physiological processes, including stress response, reproduction, acid-base regulation, growth and reproduction (Fukamachi and Meyer, 2007; Vega-Rubín de Celis et al., 2004).

Silver catfish (*Rhamdia quelen*) is a Neotropical silver catfish that shows a high potential for aquaculture in South Brazil, due to an elevated growth rate, good carcass yield, and easy reproductive handling under the subtropical climate (Baldisserotto, 2009; de Amorin et al., 2009). This species has been intensively cultured and has even been used as a model to improve the management of several fish of this family; this knowledge might be useful to silver catfish production (Barcellos et al., 2010).

Several studies have analyzed the effects of a simple stressor on fish energy metabolism (Barcellos et al. 2010, 2011; Polakof et al., 2006; Sangiao-Alvarellos et al., 2005a). Nevertheless, this study was designed to investigate the effects of different stocking densities and feeding regimes on biochemical and physiological parameters in plasma and liver as well as the expression of pituitary hormones (PRL, GH and SL) in silver catfish.

2. Materials and methods

2.1. Experimental procedures

Silver catfish (173.20 ± 8.33 g and 27.17 ± 0.55 cm) were acquired from the Fish Culture Laboratory at the Federal University de Santa Maria (southern Brazil) and transferred to the Fish Physiology Laboratory. Prior to the experiment, fish were maintained for at least 10 days in tanks of 250 L with continuously aerated and running water under a natural photoperiod (12 h light and 12 h dark). Throughout the acclimation and experimental periods, the water quality was as follows: temperature:

22.0 ± 0.7 °C; pH, 7.0 ± 0.5; dissolved oxygen levels, 8.0 ± 0.2 mg/L; nitrite, 0.08 ± 0.01 mg/L; alkalinity, 37.0 ± 3.2 mg/L $CaCO_3$ and total ammonia nitrogen 0.009 ± 0.001 mg/L.

After the acclimation period, fish were transferred to 250 L tanks with continuously aerated and running water. Specimens (n = 128) were randomly assigned to four experimental groups, in duplicate, under the following experimental conditions: (1) fed fish under high stocking density (32 kg/m^3, HSD); (2) fed fish under mean stocking density (16 kg/m^3, MSD); (3) fed fish under low stocking density (8 kg/m^3, LSD); (4) food-deprived fish under low stocking density (8 kg/m^3, LSD-FD). Fish were fed once daily at 1 % body mass whereas the starved fish were food-deprived during all the time that experiment lasted. After 14 days from the start of the experiment, fish were anesthetized with 50 mg/L eugenol for 3 min, weighed, measured and sampled (n=8 samples per experimental condition). Blood was collected from caudal vein using heparinized syringes and the plasma obtained by centrifugation of whole blood (3 min, 10 000 g, 4° C) and stored at -80 °C until analysis. Fish were then euthanized by spinal section and pituitary and liver were removed. The liver was weighed separately for hepato-somatic index (HSI) and immediately frozen in liquid nitrogen. The tissues were stored at -80 °C for posteriors analysis. The methodology of this experiment was approved by the Ethics Committee on Animal Experimentation at UFSM under registration number 24/2007 for the use of laboratory animals.

2.2. Plasma determinations

Plasma osmolality was measured with a vapour pressure osmometer (Fiske, 110 Osmometer, Norwood Massachussetts, USA). Glucose, triglycerides and lactate were measured using commercial kits from Spinreact (Glucose-HK, ref. 1001200; Lactate, ref. 10013300; TAG, ref. 10013110). Total proteins were determined using bicinchoninic acid with a commercial Thermo kit (Pierce BCA protein assay kit, ref. 23225, Thermo Scientific, USA) using bovine serum albumin as standard. Total α-amino acid levels were assessed colorimetrically using the nynhidrin method described by Moore (1968) and adapted to microplates, and using L-Alanine (Sigma, ref. A-7469) as standard. All assays were performed using a Bio-Tek PowerWave 340 Microplate spectrophotometer (Bio-Tek Instruments, Winooski, VT, USA) using KCjunior Data Analysis Software for Microsoft Windows XP.

Plasma cortisol levels (expressed in ng/mL) were measured (in duplicate) by indirect enzyme immunoassay (ELISA) as previously described in Rodríguez et al. (2000) for testosterone. Steroids were extracted as described by Baldisserotto et al. (2014). The standards and extracted plasma samples were run in duplicate. The standard curve was run from 2.50 ng/mL to 9.77 pg/mL (R^2= 0.991). The lower limit of detection (90.80 % of binding, ED 90.80) was 19.55 pg/mL. The percentage of recovery was 95%. The inter- and intra-assay coefficients of variation (calculated from the duplicate samples) were 2.23 ± 0.24% and 2.88 ± 0.34%, respectively. Cross-reactivity information for specific antibodies with intermediate products involved in steroid synthesis was furnished by the supplier (cortexolone (1.6%), 11-deoxycorticosterone (0.23%), 17-hydroxyprogesterone (0.23%), cortisol glucurinoide (0.15%), corticosterone (0.14%), cortisone (0.13%), androstenedione (<0.01%), 17-hydroxypregnenolone (<0.01%), testosterone (<0.01%)).

2.3. Liver metabolites

Frozen liver was finely minced in an ice-cold Petri dish, homogenized using an Ultra-Turrax T25 basic (IKA-Werke) with 7.5 volume of ice-cooled 0.6 N perchloric acid, neutralized (using 1 M potassium bicarbonate), centrifuged (30 min at 3220 g, 4 ºC in an Eppendorf Centrifuge 5810R) and the supernatant was used to assay the tissue metabolites. Prior to the centrifugation one aliquot was removed and frozen at -80 ºC for the analysis of triglycerides with a commercial kit (as described before). Tissue glycogen concentration was assessed using the method of Keppler and Decker (1974). Glucose obtained after glycogen breakdown (after subtracting free glucose levels), as well as total α-amino acid levels was determined as described before for plasma samples.

2.4. Liver metabolic enzyme activities

Frozen liver was finely minced in an ice-cold Petri dish, homogenized by ultrasonic disruption (Misonix inc., Microson Ultrasonic liquid processor XL-2000) with 10 volume of ice-cold stopping-buffer containing: 50 mM imidazole (Sigma I-0125) (pH 7.5), 1 mM mercaptoethanol (Sigma M-3148), 50 mM NaF (Merck ref. 1.06449), 4 mM EDTA (Sigma ED2SS), 0.5 mM PMSF (Sigma P-7626) and 250 mM

sucrose (Sigma S-9378). The homogenate was centrifuged at 10 000 g, 30 min, 4 ºC (Centrifuge 5810R, Eppendorf) and the supernatant was immediately frozen using dry ice and kept at -80 ºC until enzyme assays. Enzyme activities were determined using a Bio-Tek PowerWave 340 Microplate spectrophotometer (Bio-Tek Instruments, Winooski, VT, USA) using KCjunior Data Analysis Software for Microsoft Windows XP. Reaction rates of enzymes were determined by the increase or decrease in absorbance of NAD(P)H at 340 nm. The reactions were started by the addition of homogenates (15 μL) in duplicate, at a pre-established protein concentration, omitting the substrate in control wells (final volume of 275-295 μL, depending on the enzyme tested), and allowing the reactions to proceed at 37 ºC. The specific conditions for the enzymes hexokinase (HK, EC 2.7.1.11), glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49), fructose-biphosphatase (FBP, EC 3.1.3.11), glutamate dehydrogenase (GDH, EC 1.4.1.2), glycerol-3-phosphate dehydrogenase (G3PDH, EC 1.1.1.8), L-lactate dehydrogenase (LDH, EC 1.1.1.27), pyruvate kinase (PK, EC 2.7.1.40), phosphorilase (total and active GPase, EC 2.4.1.1), alanine transaminase (ALT, EC 2.6.1.2), aspartate transaminase (AST, EC 2.6.1.1) were previously described (Laiz-Carrión et al., 2003; Polakoff et al., 2006; Sangiao-Alvarellos et al., 2003, 2005a, 2005b) and enzymatic analyses were carried out at conditions meeting requirements for optimal velocities for *R. quelen* (results not shown). Protein levels were assayed by triplicate as in plasma samples.

2.5. Total RNA isolation

Total RNA was isolated from complete pituitaries using NucleoSpin®RNA XS kit (Macherey-Nagel) and the on-column RNase-free DNase digestion, according to manufacturer's protocol. The amount of RNA was spectrophotometrically measured at 260 nm with a BioPhotometer Plus (Eppendorf) and its quality was determined in a 2100 Bioanalyzer using the RNA 6000 Nano Kit (Agilent Technologies). Only samples with a RNA Integrity Number (RIN) higher than 9.0 were used for qPCR.

2.6. Quantification of mRNA expression levels

Firstly, attending to the previous work described by Baldisserotto et al. (2014), the concentration of 500 ng of total RNA was used to synthesize the first strand cDNA

by reverse transcription (RT) reaction using qSCRIPT™ cDNA Synthesis Kit (Quanta BioSciences). The real-time PCR was carried out with Fluorescent Quantitative Detection System (Eppendorf Mastercycler ep realplex 2 S). Each reaction mixture (10 μL) contained 4 μL at 250 pg/μL of cDNA (1 ng per reaction), 0.5 μL at 200 nM of each specific forward and reverse primers, and 5 μL of PerfeCTa SYBR® Green FastMix™ (Quanta BioSciences).

Primers used for GH were obtained from complete sequence of *R. quelen* available in GenBank (accession number EF101341), and the others for PRL and SL were obtained from Baldisserotto et al. (2014) (accession number PRL: KC195971; accession number SL: KC195972). The nucleotide sequences of specific primers are shown in Table 1. The PCR profile was as follows: (95ºC, 10 min; [95ºC, 30 sec; 60ºC, 45 sec] X 40 cycles; melting curve [60ºC to 95ºC, 20 min], 95ºC, 15 sec). The melting curve was used to ensure that a single product was amplified and to check for the absence of primer-dimer artifacts. Results were normalized to β-actin (acc. no. KC195970), owing its low variability (less than 0.25 CT) under our experimental conditions. Relative gene quantification was performed using the ΔΔCT method (Livak and Schmittgen, 2001).

2.7. Statistics

Normality and homoscedasticity were analyzed through the Kolmogorov-Smirnoff and Levene tests, respectively. Statistical differences between groups (HSD, MSD and LSD) were analyzed and detected with a one-way analysis of variance (ANOVA) test. Logarithmic transformations of the data were made when necessary to fulfill the conditions of the analysis of variance but data are shown in their decimal values for clarity. Post-hoc comparisons were made using a Tukey test. Differences between LSD and LSD-FD were analyzed by Student's T test. The differences were considered to be statistically significant at $p<0.05$.

3. Results

No mortality was observed in any group of fish throughout the 14 days of experiment. There were no significant differences in weight or length between the treatments. Differences for liver weight were detected in the HSD group that presented

the highest values, while the LSD showed a decrease in this parameter. Food deprivation during 14 days decreased liver weight and HSI in silver catfish (Table 2).

Fish held at different stocking densities and feed deprived did not show differences in plasma osmolality, glucose, lactate, protein and amino acids at the end of experimental period. Moreover, triglycerides and cortisol decreased significantly in LSD-FD after 14 days when compared to the LSD group (Table 3).

At hepatic level, glucose and glycogen decreased significantly in the LSD-FD when compared to the LSD group. Liver glycogen was significantly higher in the HSD than LSD group. Triglycerides showed maximum values at MSD. Furthermore, feed deprivation decreased triglycerides compared to LSD. Differences between treatments were not registered for liver amino acids (Table 4).

The HK, FBPase and G6PDH liver activities did not show alterations among groups (Figure 1A, 1B and 1D). The PK activity was the highest in the HSD group, presenting its minimum values of activity in the MSD group (Figure 1C). The active GPase activity showed lowest values in the MSD, and increased its activity in the fasted group (Figure 1E). The total GPase activity showed no change between groups except for the fasted group, which revealed an increase when compared to the LSD group (Figure 1F).

The ALT activity in liver did not change between groups (Figure 2A). AST activity was lower in HSD, while the activity increased in the LSD-FD group when compared to LSD (Figure 2B). The GDH activity of the HSD group was the highest of all the groups tested (Figure 2C). The G3PDH activity was lowest in the HSD group (Figure 3A). On the other hand, the LDH activity did not change between groups (Figure 3B).

Pituitary PRL expression did not change significantly between groups (Figure 4A). GH expression was lower in HSD in relation to LSD group, and was higher in the LSD-FD when compared to LSD (Figure 4B). SL showed lower values in the HSD and MSD, as well LSD-FD in relation to LSD group (Figure 4C).

4. Discussion

The stocking density used in the aquaculture activity is a very important factor for the cultivation of fish, because inadequate densities can lead to a situation of stress, affecting their behavior and physiology (Ellis et al., 2002; Montero et al., 1999). The

results from this study did not show significant differences among the stocking densities used related to weight and length parameters due to the short experimental period. Similar observations were described by North et al. (2006) where juvenile rainbow trout (*Oncorhynchus mykiss*) with a mean of 180 g body weight, maintained at densities of 9.8, 36.6 and 70.2 kg/m^3, showed no significant differences in growth between all the groups. On the other hand, liver weight showed higher values in HSD and MSD groups, which may indicate an increase in reserves at high and medium density groups, whereas the group at low density and fasting showed a decrease of this parameter. Thus, food deprivation maintained for 14 days decreased the liver weight and also the HSI, which may result from a mobilization of hepatic reserves to maintain homeostasis leading to a stressful condition for the animals. Polakof et al. (2006) also observed the decrease in HSI in gilthead sea bream (*Sparus aurata*) after 14 days under fasting conditions.

Plasma glucose, lactate, protein and amino acids were not significantly different among groups. On the other hand, a significant decrease in plasma triglycerides levels was observed in fish from LSD-FD group. Costas et al. (2011) and Polakof et al. (2006) also observed a decrease in triglycerides levels in gilthead sea bream and Senegalese sole (*Solea senegalensis*) after 14 and 21 days, respectively, of food deprivation, suggesting that this condition affect the metabolic pathways related to lipid metabolism and the mobilization of triglycerides, in order to cope with the increased energy demand imposed by this stress situation.

Glucose is a good indicator of physiological disorders resulting from different types of stressors, and may be the main source of energy used by the fish when the conditions are unfavorable (Brandão et al., 2004). In the present study no significant differences were observed between densities for liver glucose concentration, demonstrating that none of the densities were physiologically stressful for the silver catfish, but feed deprivation decreased liver glucose that may indicate a situation of stress. Thus, this species was able to maintain the glycidic homeostasis when cultured the different stocking densities studied. In the LSD group the hepatic glucose levels could have been maintained through glycolysis, while for HSD group the gluconeogenesis may have been responsible for maintenance of the hepatic glucose.

Additionally, glycogen concentration was higher in HSD and MSD groups and lower in LSD-FD when compared to LSD group. Glycogen is the form of carbohydrate stored in the liver and, when required, is enzymatically broken down and transported to

the extra hepatic tissues as glucose, being generally the first substrate to be used during fasting (Navarro and Gutiérrez, 1995). This fact could explain the decrease in liver glycogen after 14 days at the LSD-FD group, suggesting an enhancement of the energy requirements of the liver through increased glycogenolysis during the first days of fasting. The lowest HSI is observed in fish maintained at LSD-FD, thus could be partially due to a reduction in hepatic glycogen content in this group.

In addition to the decreased glucose and glycogen in liver of fish of the LSD-FD group, a decrease in liver triglycerides was also observed. The decrease in liver triglycerides concentration during food deprivation suggests a degradation of hepatic lipids. The reduction of glucose, glycogen and triglycerides could indicate the use of these metabolites in order to fuel metabolic activities. Food deprivation is a strong stressor that reduced the energetic sources as glycidic and lipid reserves that were consumed in this organ. On the other hand, an increase in hepatic triglycerides values in the MSD group was observed indicating an accumulation of energy reserves in the form of lipids in this group of fish.

The increased capacity of using glucose in the HSD group is apparently related to stored glucose in the form of glycogen, as demonstrated by increased glycogen levels in this group, and to be increasingly used through gluconeogenesis as suggested by the elevation of PK activity. However, the LSD group (fed) presented a relatively high PK activity in the liver. This could be explained by an increase of the glycolysis potential as seen with the high active GPase or the G3PDH activities. Moreover, an enhanced glycogenolytic potential was observed in liver of fish in the LSD-FD group based on decreased glycogen levels and increased GPase activity (both total activity and the percentage of the enzyme in the active form). A similar increase of glycogenolytic potential in the liver was obtained in the gilthead sea bream after being fasted for a 14 days period (Sangiao-Alvarellos et al., 2005a).

In addition, the highest values of the GDH activity in the liver were found on the fish stocked at high density. The increase of this enzyme may be related i) to the removal of excess nitrogen nutrients due to an intense metabolism, aimed to anabolic routes where new metabolic stores were incorporated, leading to the production of numerous nitrogen ammonia residues; or ii) to *de novo* synthesis of amino acids using the leftovers of ammonia, wasting energy in this process, but did not seem to be a problem in this group. The enzyme AST, that indicates acute destruction of liver, heart

and/or skeletal muscle tissues (Ohgami et al., 2007) presented the lowest values in the liver of the HSD group, pointing to better conditions in the hepatocytes in relation to the other experimental groups.

The enzyme G3PDH catalyzes the oxidation of glycerol-3-phosphate into dihydroxyacetone phosphate. Thus, in the groups of low density (LSD and LSD-FD) a production of glucose from fatty acids and triglycerides is suggested. In the MSD group this glycerol produced triglycerides and phospholipids, increasing the liver lipid reserves. On the other hand, in the HSD group, the lowest activity of G3PDH enzyme indicates that the metabolism is focused on other metabolic groups such as carbohydrates or proteins, since the triglycerides levels in the liver appear to be lower than that in the MSD group, while glycogen stores are increased in HSD.

The stocking density and food deprivation are two factors that can also influence the expression of PRL, GH and SL (Laiz-Carrión et al., 2009). Variations in PRL levels have been previously described in response to confinement stress in other fish species such as *Oncorhynchus kisutch* and *Oreochromis mossambicus* (Auperin et al., 1995; Avella et al., 1991). Previous studies in the sea bream reported an increase in the PRL mRNA expression under high density and food deprivation conditions (Laiz-Carrión et al., 2009), suggesting an activation of the expression of this hormone in stress situations and their participation in these processes. Even though, our results did not show changes in the expression of PRL in any of the groups analyzed, indicating that this hormone does not have a role in the stress processes under those conditions used in silver catfish. Thus, the stocking densities used in this study and the fasting conditions are not enough to activate the expression of PRL to mediate the stress response and its regulation.

GH has also been associated with stress process (Rotllant et al., 2000). The present study observed lower values of GH expression in the HSD group, although its values were enhanced in the LSD-FD group. Other authors also observed increased expression of GH in fish maintained in situations of food deprivation (Mingarro et al., 2002; Peréz-Sánchez and Le Bail, 1999). Moreover, as GH stimulates intermediate metabolism, the increase of the expression of this hormone in fish on fasting may be an indirect response in the regulatory role of this hormone resulting from the increasing energy demand produced by this kind of stress (Sangiao-Alvarellos et al., 2006; Wendelaar Bonga, 1997).

In the case of SL, situations of stress as confinement produces increase in the expression levels of the hormone (Wendelaar Bonga, 1997). Laiz-Carrión et al. (2009) observed in gilthead sea bream maintained under lower conditions of feed and high density an increase in SL expression levels. In contrast, our results show a decrease in expression of SL in the groups MSD and HSD. Furthermore, SL levels decreased in the LSD-FD group compared to those specimens maintained under LSD. Thus, a negative effect of the density and food deprivation was observed on the expression of SL.

5. Conclusions

In this study, food deprivation elicits metabolic changes in *R. quelen*, as seen for other fish species (Costas et al., 2011; Laiz-Carrión et al., 2012; Polakof et al., 2006). Moreover, stocking density in silver catfish evokes metabolic differences between groups, determined by behavior that causes better growth rates and higher liver stores in those animals maintained at HSD after a period of 14 days. In contrast, fish kept at LSD seem to be under a stress situation that increases glycolytic and glycogenolytic pathways. In addition, pituitary hormones showed different behavior against two stressors (stocking density and food deprivation). Therefore, further research is needed in order to establish how these metabolic pathways are activated or deactivated, with particular emphasis on different developmental phases of *R. quelen.*

Acknowledments

We would like to thank the financial support by CAPES (Ministry of Education of Brazil, Brazil) through the program PDSE-CAPES (Programa Institutional de Bolsas de Doutorado Sanduíche no Exterior - Process: 1585/12-6) to the student C.M and CNPq research fellowships to V.L.L and B.B. and by the project AGL2010-14876 (Ministry of Science and Education, Spain) to J.M.M and program FPU (Formación de Profesorado Universitario- Ref.AP2008-01194) (Ministry of Science and Education, Spain) to JAM-S.

References

Auperin, B., Rentier-Delrue, F., Martial, J.A., Prunet, P., 1995. Regulation of gill prolactin receptors in tilapia (*Oreochromis mossambicus*) after a change in salinity or hypophysectomy. J. Endocrinol. 145, 213–220.

Arjona, F.J., Ruiz-Jarabo, I, Vargas-Chacoff, L, del Río, M.P.M., Flik, G, Mancera, J.M, Klaren, P.H.M., 2010. Acclimation of *Solea senegalensis* to different ambient temperatures: implications for thyroidal status and osmoregulation. Mar. Biol. 157, 1325-1335.

Avella, M., Schreck, C.B., Prunet, P., 1991. Plasma prolactin and cortisol concentrations of stressed coho salmon, *Oncorhynchus kisutch*, in fresh water or salt water. Gen. Comp. Endocrinol. 81, 21–27.

Baldisserotto, B., 2009. Piscicultura continental no Rio Grande do Sul: situação atual, problemas e perspectivas para o futuro. Ciênc. Rural 39, 291-299.

Baldisserotto, B., Martos-Sitcha, J.A., Menezes, C.C., Toni, C., Prati, R.L., Garcia, L.O., Salbego, J., Mancera, J.M., Martínez-Rodríguez, G., 2014. The effects of ammonia and water hardness on the hormonal, osmoregulatory and metabolic responses of the freshwater silver catfish *Rhamdia quelen*. Aquat. Toxicol. *In press*, DOI: http://dx.doi.org/doi:10.1016/j.aquatox.2014.04.023.

Barcellos, L.J.G., Marqueze, A., Trapp, M., Quevedo, R.M., Ferreira, D., 2010. The effects of fasting on cortisol, blood glucose and liver and muscle glycogen in adult jundiá *Rhamdia quelen*. Aquaculture 300, 231-236.

Barcellos, L.J.G., Volpato, G.L., Barreto, R.E., Coldebella, I., Ferreira, D., 2011. Chemical communication of handling in fish. Physiol. Behav. 103, 372-375.

Barton, B.A., 2002. Stress in fishes: A diversity of responses with particular reference to changes in circulating corticosteroids. Integ. Comp. Biol. 42, 517-525.

Brandão, F.R., Gomes, L.C., Chagas, E.C., de Araújo, L.D., 2004. Densidade de estocagem de juvenis de tambaqui durante a recria em tanques-rede. Pesq. Agropec. Bras. 39, 357-362.

Costas B., Aragão C., Mancera J.M., Dinis M.T., Conceição L.E.C. (2008). High stocking density induces crowding stress and affects amino acid metabolism in Senegalese sole *Solea senegalensis* (Kaup 1858) juveniles. Aquac. Res. 39, 1-9.

457 Costas, B., Aragão, C., Ruiz-Jarabo, I., Vargas-Chacoff, L., Arjona, F., Dinis, M.T.,
458 Mancera, J., Conceição, L. (2011). Feed deprivation in Senegalese sole (*Solea*
459 *senegalensis* Kaup, 1858) juveniles: effects on blood plasma metabolites and free
460 amino acid levels. Fish Physiol. Biochem. 37, 495-504.
461 de Amorin, M.P., Gomes, B.V.C., Martins, Y.S., Sato, Y., Rizzo, E., Bazzoli N., 2009.
462 Early development of the silver catfish *Rhamdia quelen* (Quoy & Gaimard, 1824)
463 (Pisces: Heptapteridae) from the São Francisco River Basin, Brazil. Aquac. Res. 40,
464 172- 180.
465 Ellis, T., North, B., Scott, A.P., Bromage, N.R., Porter, M, Gadd, D., 2002. The
466 relationships between stocking density and welfare in farmed rainbow trout. J. Fish
467 Biol. 61, 493-531.
468 Fukamachi, S., Meyer, A., 2007. Evolution of receptors for growth hormone and
469 somatolactin in fish and land vertebrates: lessons from the lungfish and sturgeon
470 orthologues. J. Mol. Evol. 65, 359-372.
471 Herrera, M., Vargas-Chacoff, L., Hachero, I., Ruiz-Jarabo, I., Rodiles, A., Navas, J.I.,
472 Mancera, J.M., 2009. Physiological responses of juvenile wedge sole *Dicologoglossa*
473 *cuneata* (Moreau) to high stocking density. Aquac. Res. 40, 790-797.
474 Herrera, M., Ruiz-Jarabo, I., Hachero, I., Vargas-Chacoff, L., Amo, A., Mancera, J.M.,
475 2012. Stocking density affects growth and metabolic parameters in the brill
476 (*Scophthalmus rhombus*). Aquac. Int. 20 (6), 1041-1052.
477 Keppler, D., Decker, K., 1974. Glycogen. Determination with amyloglucosidase, in:
478 Bergmeyer, H.U. (Eds), Methods of Enzymatic Analysis. Academic Press, New
479 York, pp. 127–1131.
480 Laiz-Carrión, R., Martín del Río, M.P., Míguez, J.M., Mancera, J.M., Soengas, J.L.,
481 2003. Influence of cortisol on osmoregulation and energy metabolism in gilthead sea
482 bream *Sparus aurata*. J. Exp. Zool. 298A, 105–118.
483 Laiz-Carrión, R., Fuentes, J., Redruello, B., Guzmán, J.M., Martín del Río, M.P.,
484 Power, D., Mancera, J.M., 2009. Expression of pituitary prolactin, growth hormone
485 and somatolactin is modified in response to different stressors (salinity, crowding
486 and food deprivation) in gilthead sea bream *Sparus auratus*. Gen. Comp. Endocrinol.
487 162, 293-300.

488 Laiz-Carrión, R., Viana, I.R., Cejas, J.R., Ruiz-Jarabo, I., Jerez, S., Martos, J.A.,
489 Eduardo, A.B., Mancera, J.M., 2012. Influence of food deprivation and high stocking
490 density on energetic metabolism and stress response in red porgy, *Pagrus pagrus* L.
491 Aquac. Int. 20, 585-599.
492 Li, D., Liu, Z., Xie, C., 2012. Effect of stocking density on growth and serum
493 concentrations of thyroid hormones and cortisol in Amur sturgeon, *Acipenser*
494 *schrenckii*. Fish Physiol. Biochem. 38, 511-520.
495 Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using
496 real-time quantitative PCR and the 2-ΔΔCT method. Methods 25, 402-408.
497 Mancera, J.M., McCormick, S.D., 2007. Role of prolactin, growth hormone, insuline-
498 like growth factor and cortisol in teleost osmoregulation, in: Baldisserotto, B.,
499 Mancera, J.M., Kapoor B.G. (Eds.), Fish Osmoregulation, Science Publishers. pp.
500 497–515.
501 Manzon, L.A., 2002. The role of prolactin in fish osmoregulation: a review. Gen. Comp.
502 Endocrinol. 125, 291-310.
503 Mingarro, M., Vega-Rubin de Celis, S., Astola, A., Pendón, C., Valdivia, M.M., Pérez-
504 Sanchez, J., 2002. Endocrine mediators of seasonal growth in gilthead seabream
505 (*Sparus aurata*): the growth hormone and somatolactin paradigm. Gen. Comp.
506 Endocrinol. 128, 102-111.
507 Moore, S., 1968. Amino acids analysis: Aqueous dimethyl sulfoxide as solvent for the
508 ninhydrin reaction. J. Biol. Chem. 243, 6281-6283.
509 Montero, D., Izquierdo, M.S., Tort, L., Robaina, L., Vergara, J.M. 1999. High stocking
510 density produces crowding stress altering some physiological and biochemical
511 parameters in gilthead seabream, *Sparus aurata*, juveniles. Fish Physiol. Biochem.
512 20, 53-60.
513 Navarro, I., Gutiérrez, J., 1995. Fasting and starvation, in: Hochachka, P.W.,
514 Mommsen, T.P. (Eds.), Metabolic Biochemistry, Biochemistry and Molecular
515 Biology of Fishes. Elsevier, Amsterdam, pp 392-434.
516 North, B.P., Turnbull, J.F., Ellis, T., Porter, M.J., Migaud, H., Bron, J., Bromage, N.R.,
517 2006. The impact of stocking density on the welfare of rainbow trout (*Oncorhynchus*
518 *mykiss*). Aquaculture 255, 466-479.

519 Ohgami, N., Upadhyay, S., Kabata, A., Morimoto, K., Kusakabe, H., Suzuki, H., 2007.
520 Determination of the activities of glutamic oxaloacetic transaminase and glutamic
521 pyruvis transaminase in a microfluidic system. Biosens. Bioelectron. 22, 1330-1336.
522 Pérez-Sanchez, J., Le Bail, P.Y., 1999. Growth axis as marker of nutritional status and
523 growth performance in fish. Aquaculture 177, 117-128.
524 Polakof, S., Arjona, F.J., Sangiao-Alvarellos, S., Martín del Río, M.P., Mancera, J.M.,
525 Soengas, J.L., 2006. Food deprivation alters osmoregulatory and metabolic responses
526 to salinity acclimation in gilthead sea bream *Sparus auratus*. J. Comp. Physiol. B
527 176, 441-452.
528 Rodríguez, L., Begtashi, I., Zanuy, S., Carrillo, M., 2000. Development and validation
529 of an enzyme immunoassay for testosterone: Effects of photoperiod on plasma
530 testosterone levels and gonadal development in male sea bass (*Dicentrarchus labrax*,
531 L.) at puberty. Fish Physiol. Biochem. 23, 141-150.
532 Rotllant, J., Balm, P.H.M., Ruane, N.M., Pérez-Sánchez, J., Wendelaar Bonga, S.E.,
533 Tort, L., 2000. Pituitary proopiomelanocortin-derived peptides and hypothalamus-
534 pituitaryinterrenal axis activity in gilthead sea bream (*Sparus aurata*) during
535 prolonged crowding stress: differential regulation of adrenocorticotropin hormone
536 and alphamelanocyte-stimulating hormone release by corticotropin-releasing
537 hormone andthyrotropin-releasing hormone. Gen. Comp. Endocrinol. 119,152-163.
538 Ruane, N.M., Carballo, E.C., Komen, J., 2002. Increased stocking density influences the
539 acute physiological stress response of common carp *Cyprinus carpio* (L.). Aquac.
540 Res. 33, 777-784.
541 Sakamoto, T., McCormick, S.D., 2006. Prolactin and growth hormone in fish
542 osmoregulation. Gen. Comp. Endocrinol. 147, 24-30.
543 Sangiao-Alvarellos, S., Arjona, F.J., Martín del Río, M.P., Míguez, M.P., Mancera,
544 J.M., Soengas, J.L. 2005a. Time course of osmoregulatory and metabolic changes
545 during osmotic acclimation in *Sparus auratus*. J. Exp. Biol. 208, 429-4304.
546 Sangiao-Alvarellos, S., Arjona, F.J., Míguez, J.M., Miguez, J.M., Martin del Rio, M.P.,
547 Soengas, J.L., Mancera, J.M., 2006. Growth hormone and prolactin actions on
548 osmoregulation and energy metabolism of gilthead sea bream (*Sparus auratus*).
549 Comp. Biochem. Physiol. A 144, 491-500.
550 Sangiao-Alvarellos, S., Guzmán, J.M., Laiz-Carrión, R., Míguez, J.M., Martín del Rio,
551 M.P., Mancera, J.M., Soengas, J.L., 2005b. Interactive effects of high stocking

552 density and food deprivation on carbohydrate metabolism in several tissues of
553 gilthead sea bream (*Sparus aurata* L.). J. Exp. Zool. 303, 761–775.
554 Sangiao-Alvarellos, S., Laiz-Carrión, R., Guzmán, J.M., Martín del Río, M.P., Mancera,
555 J.M., Soengas, J.L., 2003. Acclimation of *Sparus aurata* to various salinities alters
556 energy metabolism of osmoregulatory and non osmoregulatory organs. Am. J.
557 Physiol. 285, R897-R907.
558 Vega-Rubin de Celis, S., Rojas, P., Gómez-Requeni, P., Albalat, A., Gutiérrez, J.,
559 Medale, F., Kaushik, S.J., Navarro, I., Pérez-Sánchez, J., 2004. Nutritional
560 assessment of somatolactin function in gilthead sea bream (*Sparus aurata*):
561 concurrent changes in somatotropic axis and pancreatic hormones. Comp. Biochem.
562 Physiol. A 138, 533-542.
563 Wendelaar Bonga, S.E., 1997. The stress response in fish. Physiol. Rev. 77, 591-625.
564 Wunderink, Y.S., Martinez-Rodriguez, G., Yufera, M., Montero, I.M., Flik, G.,
565 Mancera, J.M., Klaren, P.H., 2012. Food deprivation induces chronic stress and
566 affects thyroid hormone metabolism in Senegalese sole (*Solea senegalensis*) post-
567 larvae. Comp. Biochem. Physiol. A 162, 317-322.
568
569
570

571 **Tables**

572

573 Table 1. Specific primers used for semi-quantitative expression of prolactin (PRL),
574 somatolactin (SL), growth hormone (GH) and beta-actin (ßact) by RT-PCR, and size
575 amplified by each pair of primers.

576

RT-PCR primers	Nucleotide sequence	Size amplified
qPCR-PRL_Fw	5'-CCTGTCTCTGGTTCGCTCTCT-3'	127 bp
qPCR-PRL_Rv	5'-GTCCTGCAGCTCTCTGGTCTT-3'	
qPCR-SL_Fw	5'-TCCAGCACGCTGAGCTGATCT-3'	111 bp
qPCR-SL_Rv	5'-AAGAGTTTCCCCCATGACCTT-3'	
qPCR-GH_Fw	5'-GGACAAACCACCCTAGACGAG-3'	116 bp
qPCR-GH_Rv	5'-TTCTTGAAGCAGGACAGCAGA-3'	
qPCR-ßact_Fw	5'-GAAGTGTGACGTCGATATCCG-3'	112 bp
qPCR-ßact_Rv	5'-CCTGAACCTCTCATTGCCAAT-3'	

577

578

579

580

581

582

583

584

585

586

587

588

589

590

591

592

593

Table 2. Effects of different stocking density and/or food-deprivation for 14 days on weight, length, liver weight and HSI of silver catfish.

	Parameters			
Groups	Weight	Length	Liver weight	HSI
LSD-FD	138.09±11.23	26.29±0.80	1.28±0.10*	0.94±0.06*
LSD	161.41±14.14	26.62±0.71	2.97±0.43b	1.82±0.18
MSD	190.03±13.27	27.62±0.68	4.31±0.82ab	2.52±0.38
HSD	206.67±13.83	27.81±0.71	5.56±0.56a	2.70±0.24

Values are means ± SEM, n=8-12 fish/group. Weight and liver weight were expressed in g, length in cm and HSI in %. HSI = liver weight/wet weight x 100. Different letters indicate significant difference among densities by one-way ANOVA ($p<0.05$) and * indicate significant difference from LSD (Student´s T test, $p<0.05$).

609 Table 3. Effects of different stocking density and/or food-deprivation for 14 days on levels of osmolality, glucose, lactate, triglycerides, and
610 protein in plasma of silver catfish.
611

	Parameters						
Groups	Osmolality	Cortisol	Glucose	Lactate	Triglycerides	Protein	Amino acids
LSD-FD	250.37±6.29	38.52±4.43*	2.21±0.22	1.46±0.29	0.95±0.19*	40.03±1.88	13.95±1.31
LSD	232.12±10.31	129.77±26.32	3.03±0.57	1.88±0.44	4.97±0.70	36.80±2.36	14.34±2.01
MSD	225.00±9.95	122.19±9.31	3.59±0.35	1.99±0.34	5.99±1.28	37.35±3.10	16.82±1.22
HSD	231.25±13.44	58.22±19.46	3.38±0.50	1.27±0.17	4.90±0.43	37.62±2.02	17.35±1.28

612 Values are means ± SEM, n=8-12 fish/group. Osmolality is expressed as mOsm/kg, cortisol as ng/mL, glucose, lactate,
613 triglyceride and amino acids are expressed as mM and protein as mg/mL. * Indicate significant difference from LSD
614 (Student´s T test, $p<0.05$).
615
616
617
618

Table 4. Effects of different stocking density and/or food-deprivation for 14 days on levels of glucose, glycogen, triglycerides, and amino acids in liver of silver catfish.

	Parameters			
Groups	Glucose	Glycogen	Triglycerides	Amino acids
LSD-FD	3.77±0.25*	4.71±0.76*	6.60±1.95*	35.51±5.98
LSD	6.89±1.13	25.45±4.27b	27.82±6.10b	61.25±14.70
MSD	6.64±1.01	52.70±12.01ab	88.50±23.18a	78.55±18.21
HSD	5.36±1.00	56.77±6.47a	39.47±6.27ab	125.26±32.19

Values are means ± SEM, n=8-12 fish/group. Glucose, glycogen, triglycerides and amino acids are expressed as µmol/g wet mass). Different letters indicate significant difference among densities by one-way ANOVA ($p<0.05$) and * indicate significant difference from LSD (Student´s T test, $p<0.05$).

628 **Figures**

629

630 Figure 1. Activities of carbohydrate metabolism enzymes HK (A), FBPase (B), PK (C),
631 G6PDH (D), total GPase (E), and active GPase (F) in liver of silver catfish at different
632 stocking density and/or food-deprivation for 14 days (means ± SEM, n=8). Different
633 letters indicate differences among groups by one-way ANOVA ($p<0.05$) and * indicate
634 significant difference from LSD (Student´s T test, $p<0.05$).

635

636 Figure 2. Activities of amino acid catabolic enzymes ALT (A), AST (B), and GDH (C)
637 in liver of silver catfish at different stocking density and/or food-deprivation for 14 days
638 (means ± SEM, n=8). Further information as detailed in Figure 1.

639

640 Figure 3. Activities of lipid metabolism enzyme G3PDH (A) and lactate metabolism
641 enzyme LDH (B) in liver of silver catfish at different stocking density and/or food-
642 deprivation for 14 days (means ± SEM, n=8). Further information as detailed in Figure
643 1.

644

645 Figure 4. mRNA expression of PRL (A), GH (B) and SL (C) in hypophysis of silver
646 catfish at different stocking density and/or food-deprivation for 14 days (means ± SEM,
647 n=8). Further information as detailed in Figure 1.

648

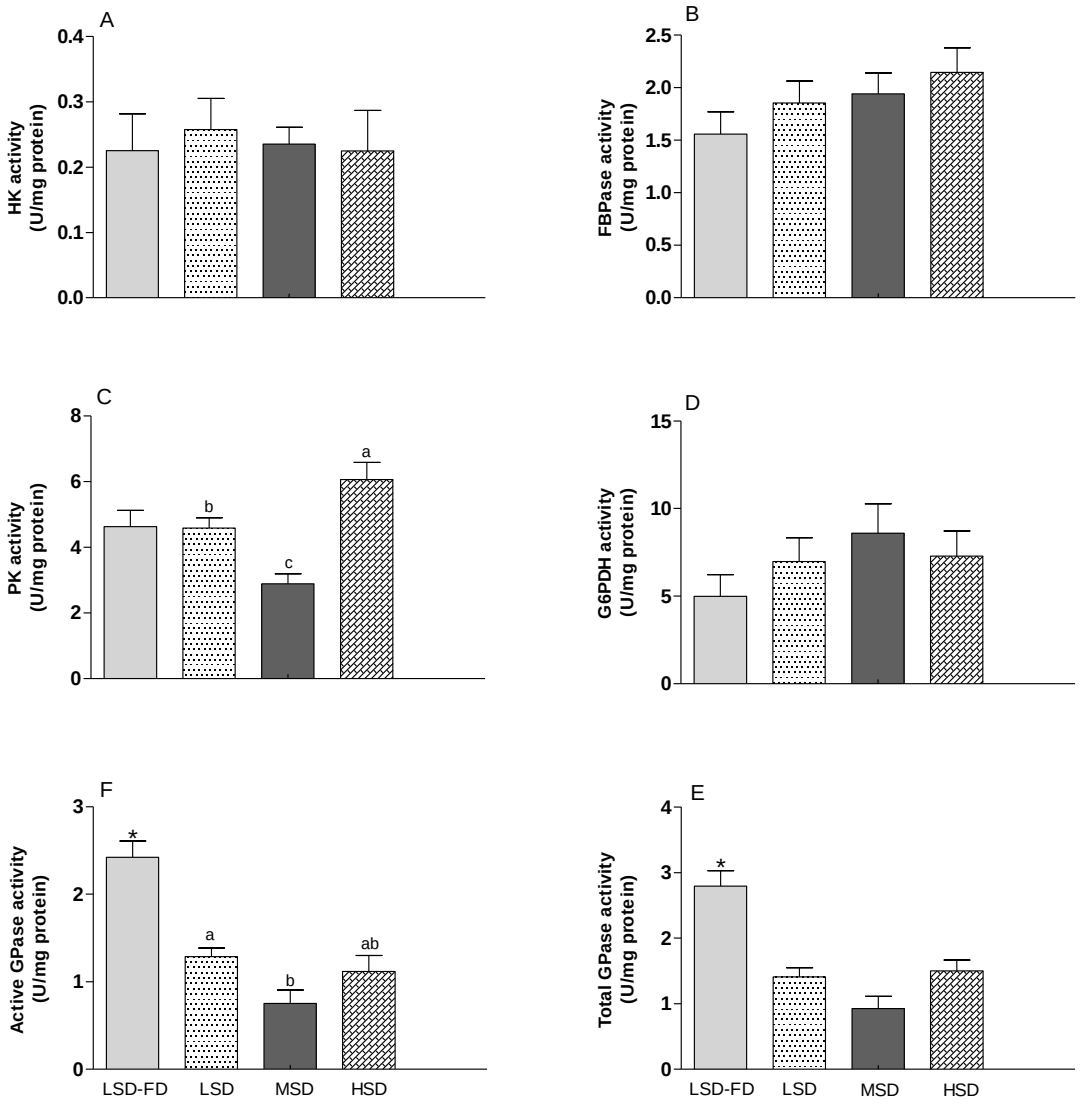
649

650

651

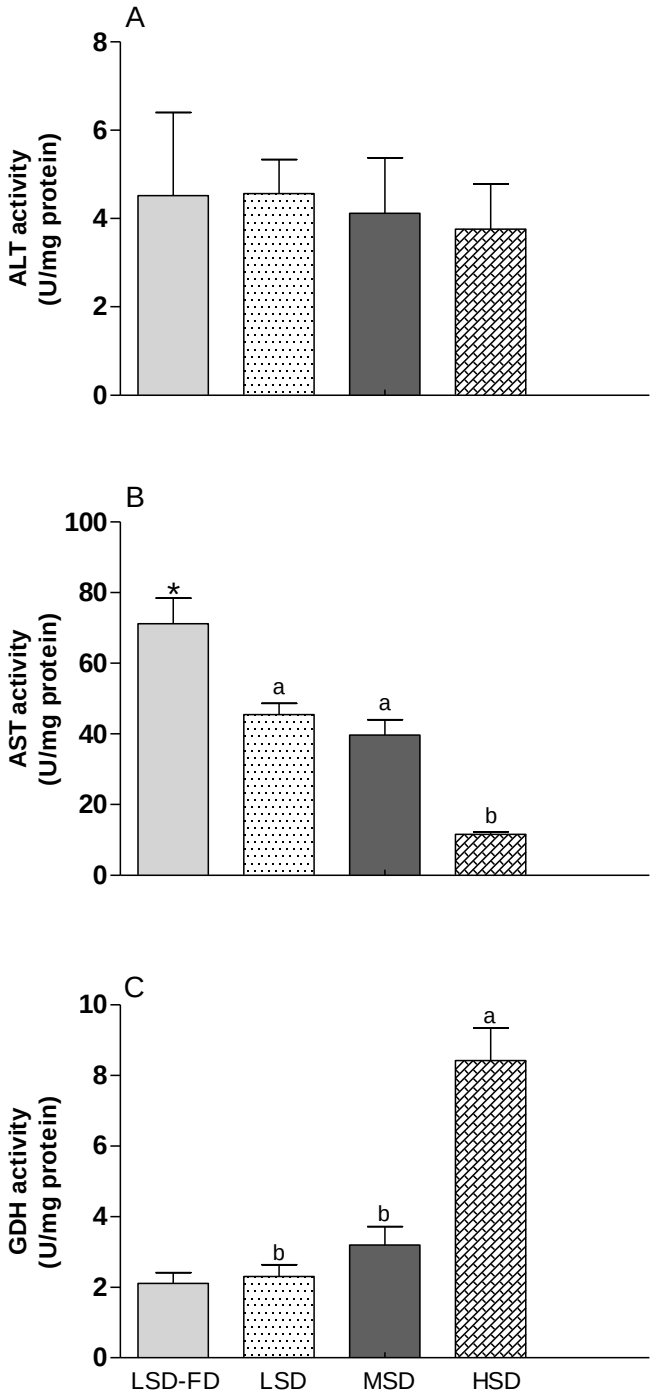
652

653



654
655

656 Figure 1



657

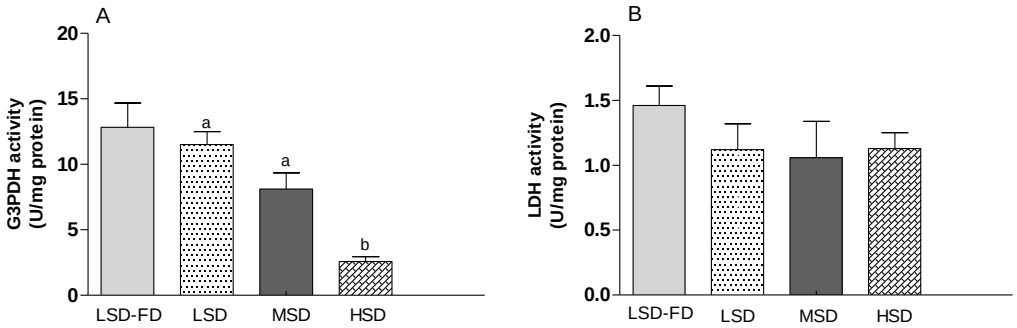
658 Figure 2

659

660

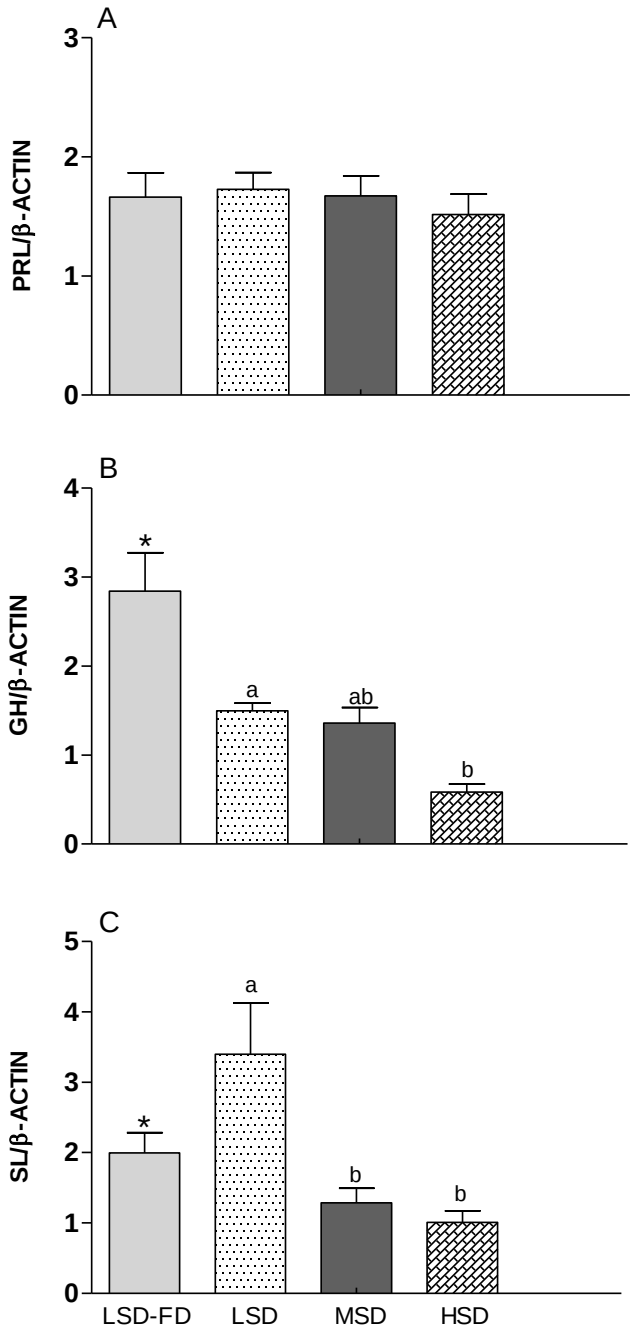
661

662



663

664 Figure 3



665

666 Figure 4