



Effect of crowding stress on growth performance, the antioxidant system and humoral immunity in hybrid sturgeon

Baoliang Bi^{a,b}, Yin Yuan^a, Yao Zhao^a, Mingyi He^a, Haoqing Song^a, Lingfu Kong^{a,b}, Yu Gao^{a,b,*}

^a College of Animal Science and Technology, Yunnan Agricultural University, Kunming 650201, China

^b Key Laboratory for Plateau Fishery Resources Conservation and Sustainable Utilization of Yunnan Province, Yunnan Agricultural University, Kunming 650201, China

ARTICLE INFO

Keywords:

Crowding stress
Growth performance
Antioxidant capacity
Innate immunity
Metabolome

ABSTRACT

To evaluate effects of crowding stress on the physiological mechanisms of juvenile hybrid sturgeon (*♀Acipenser baerii* × *♂Acipenser schrenckii*, 23 ± 1 g body weight), an experiment was assigned to three initial stocking densities in flowing water ponds (LD = 100 and 150 fish/m³; MD = 200 and 250 fish/m³; and HD = 300 and 350 fish/m³) for 60 days. Ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) was used to identify differential metabolites between different stocking densities. The final weight, weight gain rate (WGR) and specific growth rate (SGR) decreased significantly as the stocking density increased ($P < 0.05$). Gill filaments incurred injury in the MD and HD groups. Crowding stress increased significantly serum cortisol and glucose levels ($P < 0.05$), and inhibited the antioxidant capacity. However, stocking density did not affect innate immunity parameters in fish, including complement c3, immunoglobulin M (IgM) and lysozyme (LZM). These negative growth performances to crowding stress mainly involved pathways associated with ascorbate and aldarate metabolism, butanoate metabolism, and fatty acid degradation. The gluconeogenesis and glycogenolysis were activated by the glucagon signaling pathway in order to meet the increased energy expenditure of fish under stress, which played a protective role against lipid peroxidation. Assessment of these factors allows for a more comprehensive understanding of the underlying physiological mechanisms associated with crowding stress in hybrid sturgeon.

1. Introduction

The hybrid sturgeon (*♀Acipenser baerii* × *♂Acipenser schrenckii*) is one of the most popular species of sturgeon to be cultivated around the world and has low oxygen tolerance, a growth rate and strong disease resistance (Hao et al., 2021). However, with the development of intensive artificial breeding, the production of sturgeon is also faced with some key problems, including low growth rate, a deterioration of quality and the frequent occurrence of diseases; consequently, it is vital that we investigate new and healthy stocking technologies (Ni et al., 2014a, 2014b; Yang et al., 2011).

Stocking density, as a key production parameter, can improve our utilization of the aquatic environment and directly influence the economic benefits of production. However, stocking density also causes fierce competition for dissolved oxygen, food consumption and the ecological niche in aquatic environments. Previous studies have shown

that high stocking density inhibits growth and can reduce the immunity and survival rate of aquatic species; final weight, survival rate, weight gain rate, specific growth rate and feed utilization rate have all been shown to decrease significantly as stocking density increases (Çelikkale et al., 2005; Dediu et al., 2021; Long et al., 2019; Ni et al., 2014a, 2014b). Therefore, stocking density has been proven to be a chronic source of stress in sturgeon culture; it is crucial that we identify the most appropriate stocking density for the healthy aquaculture of sturgeon.

The secretion of cortisol, as an important glucocorticoid secreted by inter-renal tissues, can be rapidly regulated through the hypothalamic-pituitary-interrenal axis (HPI) when the fish is subjected to external stress or stimulation, and activate key enzymes of gluconeogenesis, such as glucose-6 phosphatase, to cope with the increased energy demand of the body under stress (Khani and Tayek, 2001). Therefore, an increase of cortisol level is often regarded as a signal of crowding stress in fish (Ramsay et al., 2006; Ray and Sinha, 2014). As liver is the main organ in

* Corresponding author at: College of Animal Science and Technology, Yunnan Agricultural University, Kunming 650201, China.

E-mail address: gaoyu@ynau.edu.cn (Y. Gao).

¹ ORCID: 0000-0003-2887-2559.

the homeostatic adjustments to stress, fish can clear excessive levels of reactive oxygen species (ROS) via their own antioxidant system; however, when fish are stressed, the rate of ROS generation exceeds the clearance rate of the antioxidant system, thus resulting in the accumulation of ROS, thus resulting in tissue damage and the production of lipid peroxides (Regoli et al., 2002). Crowding stress may also affect mortality and can exert a negative influence on the innate immune response while increasing apoptosis (Lin et al., 2018; Ortuño et al., 2001). Complement and lysozyme are regarded as an essential humoral system involved in the innate immunity of fish and are ubiquitously expressed in living organisms (Nakao et al., 2011; Saurabh and Sahoo, 2008). Metabolomics technologies have been recently applied to study the physiological response to crowding stress. For example, gut microbiome and the pentose phosphate pathway involved in the adaption of crowding stress and played potential role in the regulation of glucose metabolism in Wuchang bream (*Megalobrama amblycephala*) (Du et al., 2019). Given this, it is evident that the homeostasis of the glucocorticoid and antioxidant system systems, along with innate immunity, can experience deleterious effects when fish are placed under crowding stress; however, little is known about the specific effects of crowding stress on the physiological mechanisms controlling metabolism.

In this study, we analyzed a range of parameters associated with growth performance, stress, the antioxidant system and innate immunity in hybrid sturgeon when cultured at different stocking densities for 60 days. Changes in protein expression are known to cause amplified changes in metabolism (Taylor et al., 2002). Therefore, we also applied ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) to investigate the influence of crowding stress on key metabolites associated with growth, the antioxidant capacity and the immune system of hybrid sturgeon. In addition, metabolomics technology was used to identify metabolites as potential biomarkers of crowding stress in hybrid sturgeon. The assessment of these factors allows for a more comprehensive understanding of the mechanisms underlying the effect of crowding stress on the aquaculture of hybrid sturgeon.

2. Materials and methods

2.1. Ethics statement

The use of hybrid sturgeon (*♀Acipenser baerii* × *♂Acipenser schrenckii*) in this study complied with animal welfare laws, guidelines, and policies, as approved by the Scientific Ethics Committee of Yunnan Agricultural University, Kunming, China. To minimize the stress response to sampling, hybrid sturgeon were anesthetized with 100 mg/L tricaine methanesulfonate (MS222, Sigma-Aldrich, MO, USA) prior to all sampling techniques.

2.2. Fish culture

From August to October 2021, we obtained juvenile hybrid sturgeon from the same batch of artificially bred juveniles from Yunnan Dianze Aquaculture Co., Ltd. (Qujing, China). The hybrid sturgeon were cultured in flowing water obtained from a nearby river in ponds that were 7 m² (2.65 × 2.65 m) with a water depth of 0.35 m and an effective volume of 2.45 m³. A separate water intake was located in the center of each pond using bottom drainage; the rate of water intake was 0.546 m³/s. To prevent man-made influences, a heat preservation shed created above the ponds.

The juvenile hybrid sturgeon were fasted for 24 h before the experiment. Fish of a uniform size (body weight: 23 ± 1 g), with no deformities, no disease, and strong vitality were selected. According to the previous studies (Ni et al., 2014; Ni et al., 2016) and production requirement of aquaculture company, the fish were then randomly assigned to three groups for 60 days of culture: low density (LD, 100 and 150 fish/m³, T100 and T150, respectively), medium density (MD, 200

and 250 fish/m³, T200 and T250, respectively) and high density (HD, 300 and 350 fish/m³, T300 and T350, respectively) including 6 density groups with 3 biological replicates. Fish were fed daily every morning and evening (08:00 and 18:00) at average 1.7 % of the total weight of fish. Fish were weighed every 2 weeks to regulate the feeding rates according to the total weight of fish. We used a commercial compound feed produced by Kunming Hengda Feed Co., Ltd. (crude protein ≥ 42.0 %, crude lipid ≥ 12.0 %, crude fiber ≤ 6.0 %, crude ash ≤ 18.0 %). The ponds were inspected three times daily to observe the status of fish health. Deaths were recorded and the survival rate was calculated. Water parameters were recorded and checked daily, including non-ionic ammonia, pH, water temperature and dissolved oxygen.

2.3. Sample collection

The fish were fasted for 24 h before sampling. Total 450 fish were randomly selected for the body weight and body length measurement (75 fish for each density group). Then, we calculated the mean body weight and body length, the rate of weight gain rate and the specific growth rate for each group.

Serum samples were collected from another ten fish from each group from the caudal vein with a sterile 1 mL syringe and withdrawn into microcentrifuge tubes without anticoagulant. After standing in a refrigerator at 4 °C for 6 h, the samples were centrifuged at 4500 rpm at 4 °C for 10 min; then, we removed the supernatant which was then stored at − 80 °C for the analysis of biochemical parameters.

At the end of the experiment, livers were dissected from every 18 fish in each density group (6 fish for each pond). To prepare a 10 % tissue homogenate, we took 0.1 g of liver and 0.9 mL of 0.65 % normal saline which was then homogenized. After centrifugation at 4000 rpm at 4 °C for 10 min, the supernatant was removed and stored at − 80 °C. We subsequently pooled equal amounts of the total protein from every 6 livers sample in the same pond for the analysis of enzyme activity.

One section of the gill was stored in 4 % paraformaldehyde solution for histological analyses from every 18 fish.

2.4. Serum stress-related parameters

Serum glucose levels were measured using commercially available kits (Shanghai Rongsheng Biological Pharmaceutical Co., Ltd., Shanghai, China). The levels of cortisol and adrenocorticotrophic hormone (ACTH) in the serum were determined by commercially available ELISA kits (Jiangsu Jingmei Biotechnology Ltd., Yancheng, China). Results were determined with a microplate reader (Rayto RT-6100 system, Shenzhen, China).

2.5. Antioxidant-related parameters

The serum levels of malondialdehyde (MDA) were determined by the thiobarbituric acid method (Ohkawa et al., 1979). Superoxide dismutase (SOD), catalase (CAT), glutathione (GSH) and glutathione peroxidase (GSH-Px) activities in the liver were determined with a microplate reader (Rayto RT-6100 system, Shenzhen, China) at 550, 405, 420, 412, nm, respectively. MDA and antioxidant-related parameter detection kits (SOD, CAT, GSH and GSH-Px) were purchased from NanJing JianCheng Bioengineering Institute, Nanjing, China. Total protein levels in the supernatant was determined using a BCA protein concentration assay kit (Biosharp, Hefei, China).

2.6. Thyroid hormone measurement

The levels of serum T3, T4, free T3 (FT3), and free T4 (FT4) were determined by radioimmunoassay (XH-6080 radioimmunoassay γ counter, Xi'an, China) in accordance with the manufacturer's instructions (Beijing North Institute of Biotechnology Co., Ltd, Beijing, China). The detection limits of these assays were 0.5–8 ng/mL for T3,

20–320 ng/mL for T4, 2–32 pmol/L for FT3 and 4–64 pmol/L for FT4, respectively.

2.7. Innate immunity-related parameters

The levels of immunoglobulin M (IgM) in serum were determined by immune turbidimetry using commercially available ELISA kits (Jiangsu Jingmei Biotechnology Ltd., Yancheng, China). The levels of complement C3, C4 and lysozyme (LZM) in liver tissue were determined by commercial kits (NanJing JianCheng Bioengineering Institute, Nanjing, China). Total protein levels in the supernatant were determined using a BCA protein concentration assay kit (Biosharp, Hefei, China). Results were acquired with a microplate reader (Rayto RT-6100 system, Shenzhen, China).

2.8. Determination of the metabolomic profiles of serum samples

Serum samples from the LD (100 fish/m³, T100), MD (250 fish/m³, T250) and HD (350 fish/m³, T350) groups were selected for metabolomics analysis (N = 18). In brief, 50 µL of each serum sample was mixed with 250 µL of 20 % acetonitrile/methanol and then centrifuged at 12,000 rpm for 10 min at 4 °C. Some of the supernatants were re-centrifuged and then transferred through a Protein Precipitation Plate into an UPLC-MS/MS system (UPLC, ExionLC AD; MS, QTRAP® 6500 + System).

The analytical conditions were as follows:

T3 method : HPLC: column, Waters ACQUITY UPLC HSS T3 C18 (100 mm × 2.1 mm i.d./1.8 µm); solvent system, water with 0.05 % formic acid (A), acetonitrile with 0.05 % formic acid (B). The gradient was started at 5 % B (0 min), increased to 95 % B (8–9.5 min) and finally ramped back to 5 % B (9.6–12 min); flow rate, 0.35 mL/min; temperature, 40 °C; injection volume: 2 µL.

Amide method: HPLC: column, ACQUITY UPLC BEH Amide (i.d.2.1 × 100 mm, 1.7 µm); solvent system, water with 10 mM ammonium acetate and 0.3 % ammonium hydroxide (A), 90 % acetonitrile/water (V/V)(B); The gradient was started at 95 % B (0–1.2 min), decreased to 70 % B (8 min), 50 % B (9–11 min) and finally ramped back to 95 % B (11.1–15 min); flow rate, 0.4 mL/min; temperature, 40 °C; injection volume: 2 µL.

Linear ion trap (LIT) and triple quadrupole (QQQ) scans were acquired on a triple quadrupole-linear ion trap mass spectrometer (QTRAP) operating in both positive and negative ion modes.

All metabolites were collated by Analyst 1.6.3 software (Sciex, Framingham, MA) and quantified with Multiquant 3.0.3 software (Sciex). Principal component analysis (PCA) was performed by the statistical function 'prcomp' within the R environment (www.r-project.org). Variable importance in the projection (VIP) values were extracted from OPLS-DA data and score plots and permutation plots were generated using the R package MetaboAnalystR (V1.0.1). Significantly regulated metabolites among different density groups were determined by specific criteria: VIP > 1, $P < 0.05$, and $|\log_2\text{FC}| < 1$. The identified metabolites were then annotated and mapped to the KEGG Pathway database (<http://www.kegg.jp/kegg/pathway.html>). Pathways in which $P < 0.05$ were accepted as significantly regulated metabolites and were then mapped into metabolite sets enrichment analysis (MSEA).

2.9. Statistical analysis

Weight gain rate (WGR, %) = $100 \% \times (\text{final body weight (g)} - \text{initial body weight (g)}) / \text{initial body weight (g)}$

Specific growth rate (SGR, %) = $100 \% \times (\ln W_f - \ln W_i) / \text{feeding days}$

$\ln W_f$ = the natural logarithm of the final weight

$\ln W_i$ = the natural logarithm of the initial weight

Mortality (%) = $\text{death fish number} / \text{initial fish number} \times 100 \%$

All statistical analyses were performed using Excel 2016 (Microsoft Corp., Redmond, WA, USA) and SPSS 22.0 (IBM SPSS Statistics for Windows, Version 22.0, Armonk, NY, USA). Data are presented as mean ± standard error (S.E.). The normal distribution and variance homogeneity of measurement data were measured. Significant differences were identified by one-way analysis of variance followed by Duncan's test. Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Effects of crowding stress on growth performance

During the 60-day period of culture, there were no significant differences in terms of water parameters when compared between different stocking density groups, including non-ionic ammonia ≤ 0.1 mg/L, pH 7.4–7.8, water temperature 16 ± 1 °C and dissolved oxygen > 6 mg/L in each pond. However, there were significant differences in growth performance among the different density groups. The growth curve between final body weight and feeding days showed a simple linear regression relationship rather than a logistic regression relationship over the 60 days. The correlation equations were $Y = 0.7759X + 20.20$ ($R^2 = 0.9841$), $Y = 0.7443X + 21.70$ ($R^2 = 0.9867$), $Y = 0.7253X + 21.37$ ($R^2 = 0.9869$), $Y = 0.6627X + 21.72$ ($R^2 = 0.9833$), $Y = 0.5443X + 23.19$ ($R^2 = 0.9991$) and $Y = 0.4881X + 22.25$ ($R^2 = 0.9992$) in T100, T150, T200, T250, T300 and T350, respectively (Fig. 1).

The final body weight, WGR and SGR of the hybrid sturgeon decreased significantly as the stocking density increased over the 60-day period of culture (Table 1, $P < 0.05$). The final body weight/length, WGR and SGR were lowest after 60-days of feeding at a culture density of T350. The final body weight did not differ significantly when compared among the culture densities of T100, T150, T200 and T250. However, the mortality rate increased significantly in T350 when compared with densities of T100, T150 and T200 ($P < 0.05$).

3.2. Effects of crowding stress on gill pathology

The gills of fish from the LD group had an intact structure and the gill lamellae were uniformly arranged as a whole and all there was good integrity in terms of cellular integrity (Fig. 2). However, both the MD and HD groups showed different degrees of gill injury when compared with the LD group. As density increased, the pathological changes in the

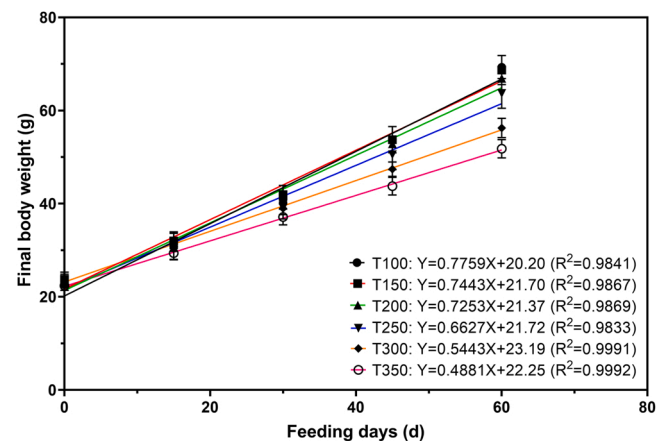


Fig. 1. Growth curve of hybrid sturgeon estimated using simple linear regression when cultured at different densities (T100, T150, T200, T250, T300 and T350) for 60 days.

Table 1

Effects of crowding Stress on growth performance and feed conversion efficiency of sturgeon.

	T100	T150	T200	T250	T300	T350
Initial body weight (g)	22.61 ± 0.40	23.74 ± 1.55	23.67 ± 0.50	23.74 ± 1.14	23.33 ± 1.10	22.39 ± 0.98
Initial body length (cm)	19.73 ± 0.36	19.6 ± 0.45	19.15 ± 0.43	19.71 ± 0.27	19.9 ± 0.44	19.69 ± 0.43
Final body weight (g)	69.31 ± 0.31a	68.7 ± 3.13a	66.91 ± 0.76a	63.68 ± 3.22a	56.25 ± 2.08b	51.81 ± 1.97c
Final body length (cm)	27.9 ± 0.26b	28.27 ± 0.33b	30.02 ± 0.50a	28.00 ± 0.30b	27.75 ± 0.34b	26.40 ± 0.34c
Weight gain rate (%)	206.73 ± 4.17a	190.21 ± 7.45b	182.86 ± 5.32 BCE	168.16 ± 2.00c	141.37 ± 4.86d	131.62 ± 4.09d
Specific growth rate (%)	1.87 ± 0.02a	1.77 ± 0.04b	1.73 ± 0.03 BCE	1.64 ± 0.01c	1.47 ± 0.03d	1.4 ± 0.03d
Mortality (%)	0.38 ± 0.13a	0.32 ± 0.11a	0.36 ± 0.12a	0.53 ± 0.02ab	0.48 ± 0.07ab	0.73 ± 0.06b

Notes: Feed ingredients were provided by Kunming Hengda Feed Co., Ltd. (crude protein $\geq 42.0\%$, crude lipid $\geq 12.0\%$, crude fiber $\leq 6.0\%$, crude ash $\leq 18.0\%$). T100 and T150 indicated the low density group (LD, 100 and 150 fish/m³); T200 and T250 indicated the medium density group (MD, 200 and 250 fish/m³); T300 and T350 indicated the high density group (HD, 300 and 350 fish/m³)

Values are presented as mean $\pm$ SE (n = 3). Values in the same column with different superscript letters are significantly different ($P < 0.05$).

Weight gain rate (WGR, %) = $100\% \times (\text{final body weight (g)} - \text{initial body weight (g)}) / \text{initial body weight (g)}$

Specific growth rate (SGR, %) = $100\% \times (\ln W_f - \ln W_i) / \text{feeding days}$, $\ln W_f$ = the natural logarithm of the final weight, $\ln W_i$ = the natural logarithm of the initial weight, feeding days between $\ln W_f$ and $\ln W_i$

Mortality (%) = $\text{death fish number} / \text{initial fish number} \times 100\%$.

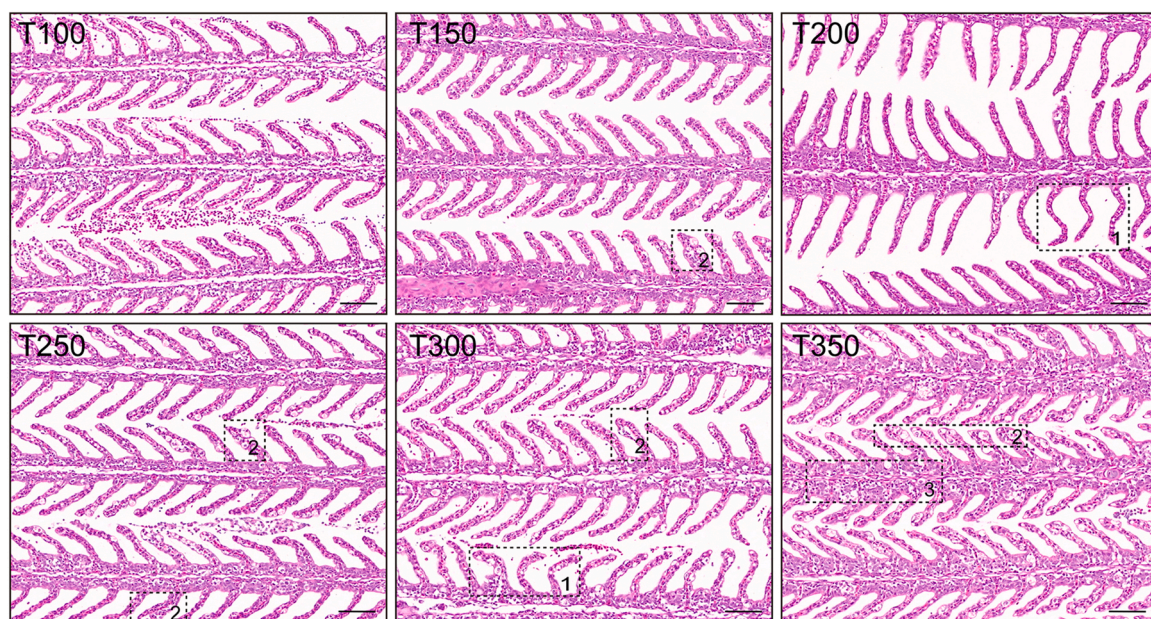


Fig. 2. Histological analysis of the gills in hybrid sturgeon cultured at different densities (T100, T150, T200, T250, T300 and T350) for 60 days. The dashed line box indicates pathological changes in the gills. (1) The gill lamellae were rod-shaped and twisted. (2) The gill lamellae were swollen on the top. (3) Adhesion and hyperplasia at the base of the gill lamellae. Scale bar = 100 μm .

gills became more obvious; the gill lamellae were rod-shaped and twisted and swollen on the top. We also observed adhesion and hyperplasia at the base of the gill lamellae.

3.3. Effects of crowding stress on the serum levels of ACTH, cortisol and glucose

As culture density increased, the serum levels of cortisol and glucose first increased and then decreased (Fig. 3A). The levels of cortisol peaked at 138.5 ± 7.62 ng/mL and 134.84 ± 7.52 ng/mL at culture densities of T250 and T300, respectively ($P < 0.05$). The levels of glucose were significantly higher in the HD group ($P < 0.05$). However, the levels of ACTH were not significantly different when compared across the different density groups.

3.4. Effects of crowding stress on thyroid hormone

The levels of T4 were higher than those of T3 in the serum from hybrid sturgeon. The serum levels of T4 and FT3 decreased significantly as crowding stress increased ($P < 0.05$). The levels of T4 and FT3 were

higher in the LD group than that in the HD group ($P < 0.05$). T3 and FT4 levels did not change significantly after crowding stress for 60 days (Fig. 3B).

3.5. Effects of crowding stress on antioxidant-related parameters

As the culture density increased, the stress caused by crowding increased and the antioxidant capacity was inhibited in the liver and serum (Fig. 4). The SOD activity, GSH-PX activity and MDA content only showed a significant change in the serum ($P < 0.05$). SOD activity in the T100 was significantly higher than that in the HD group ($P < 0.05$); GSH-PX activity was significantly higher in the T150 compared with other density groups ($P < 0.05$). CAT activity showed a significant decrease in both the serum and liver as crowding stress increased ($P < 0.05$).

However, the contents of MDA and GSH showed an increase trend as crowding stress increased ($P < 0.05$) and were highest in the T350 group.

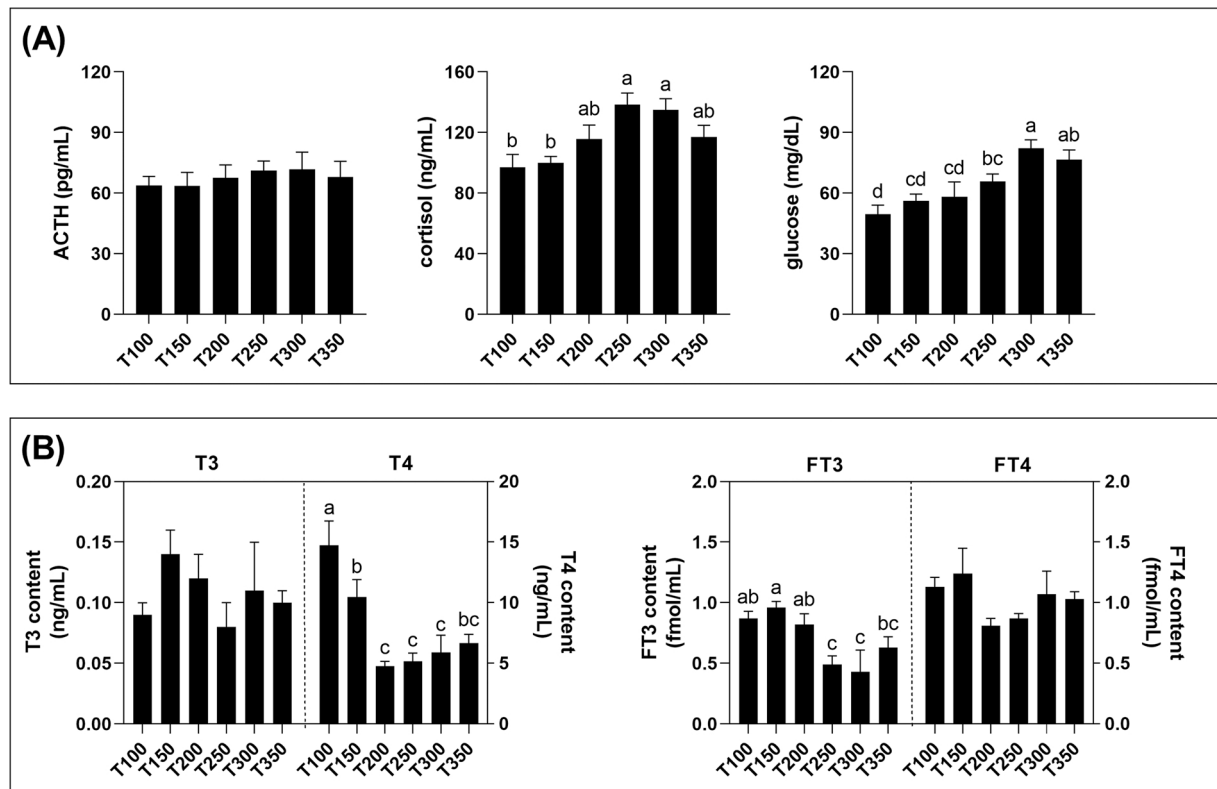


Fig. 3. Effects of crowding stress on ACTH, cortisol, glucose (A) and thyroid hormone (B) in serum of hybrid sturgeon cultured at different densities (T100, T150, T200, T250, T300 and T350) for 60 days. Values are presented as mean $\pm$ SE ($n = 3$). Values in the column with different letters are significantly different ($P < 0.05$). ACTH: adrenocorticotrophic hormone; T3: triiodothyronine; T4: thyroxine; FT3: free triiodothyronine; FT4: free thyroxine.

3.6. Effects of crowding stress on parameters related to innate immunity

As culture density increased, only C4 content showed a significant decrease in the liver ($P < 0.05$, Fig. 5). C4 content was significantly higher in the T150 than that in the HD group ($P < 0.05$). Other majority of parameters related to innate immunity, C3, IgM and LZM, in the liver and serum did not change significantly over the period of culture, thus indicating that the HD condition had no effect on the innate immunity of sturgeon at the biochemical level (Fig. S1).

3.7. Effects of crowding stress on metabolism in serum

Typical base peak chromatograms obtained by electrospray ionization are shown in Fig. S2A. PCA was performed to identify statistical differences between the LD, MD and HD groups. The differences in metabolism according to PC1 and PC2 were 36.79 % and 9.07 %, respectively (Fig. S2B). After constructing a venn for the total metabolites across the different density groups, a total of 65 different metabolites were identified; of these, 22 were significantly different for T100 vs T250, 34 for T100 vs T350 and 9 for T250 vs T350 (Fig. S2C).

Based on the KEGG IDs of the differential metabolites, crowding stress altered several different pathways: ascorbate and aldarate metabolism (ko00053), butanoate metabolism (ko00650), fatty acid degradation (ko00071), bile secretion (ko04976), citrate cycle (ko00020), the glucagon signaling pathway (ko04922) and neuroactive ligand-receptor interaction (ko04080, $P < 0.05$) when compared between T100, T250 and T350. However, tyrosine metabolism (ko00350) and the thyroid hormone signaling pathway (ko04919) were significantly enriched for T250 vs T350 (Fig. 6, $P < 0.05$).

In addition, all metabolites and different density groups in the significant metabolic pathway were clustered into two branches (Fig. 7); the lowest density (T100) was separated from the crowding stress group

(T250 and T350). The levels of L-palmitoylcarnitine and palmitoyl-ethanolamide were significantly down-regulated as density increased ($VIP > 1$, $P < 0.05$, $|\log_2FC| < 1$). However, the levels of 3-hydroxytyramine, fumaric-acid, alpha-ketoglutaric-acid, glutaric-acid, xylulose-5-phosphate, 4-aminobutyric-acid, myo-inositol, dopa, and thyroxine were up-regulated as density increased ($VIP > 1$, $P < 0.05$, $|\log_2FC| < 1$).

4. Discussion

4.1. Effects of crowding stress on growth performance

In intensive aquaculture, stocking density is considered to be one of the most important factors in determining fish survival, growth, welfare and health (Ni et al., 2014a, 2014b; Wunderink et al., 2011). In this study, the growth performance of hybrid sturgeon was negatively correlated with the crowding stress effect. These results are consistent with those of *Salvelinus fontinalis*, juvenile Russian sturgeon (*Acipenser gueldenstaedtii*) and another hybrid sturgeon (bester♀ $\times$ beluga♂) (Çelikkale et al., 2005; Dediu et al., 2021; Wafa et al., 2004). With the extension of time, crowding stress was previously found to have a negative effect on the regulation of the growth axis of Chinese sturgeon (*Acipenser sinensis*) (Long et al., 2019). In this study, the mortality at the highest density (T350) was higher than that in the LD and MD (T200) groups; these findings were consistent with those reported previously for Nile tilapia (*Oreochromis niloticus*) and *Labeo bata* (Garcia et al., 2013; Karnatak et al., 2021). However, most indices of growth performance (WGR, SGR and SR) were not significantly difference when compared between the T300 and T350 groups. The reason for this may be that the hybrid sturgeon gradually adapted to this living environment under long-term crowding stress; this is supported by the reduction in cortisol levels in the serum over time. Sturgeon species have two pairs of barbels

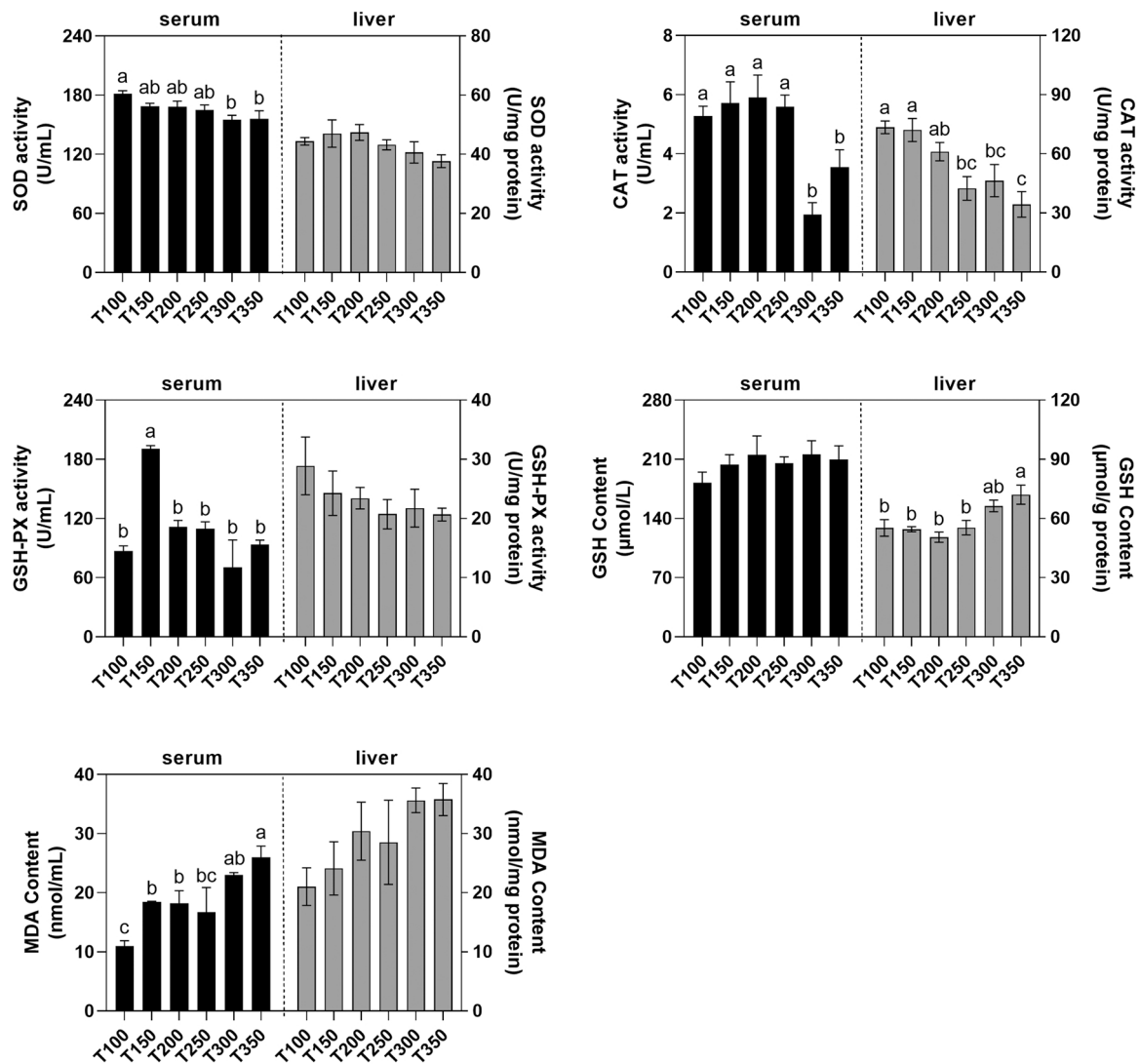


Fig. 4. Effects of crowding stress on antioxidant capacity of hybrid sturgeon cultured at different densities (T100, T150, T200, T250, T300 and T350) for 60 days. Values are presented as mean $\pm$ SE ($n = 3$). Values in the column with different letters are significantly different ($P < 0.05$). SOD: superoxide dismutase; CAT: catalase; GSH-PX: glutathione peroxidase; GSH: glutathione; MDA: malondialdehyde.

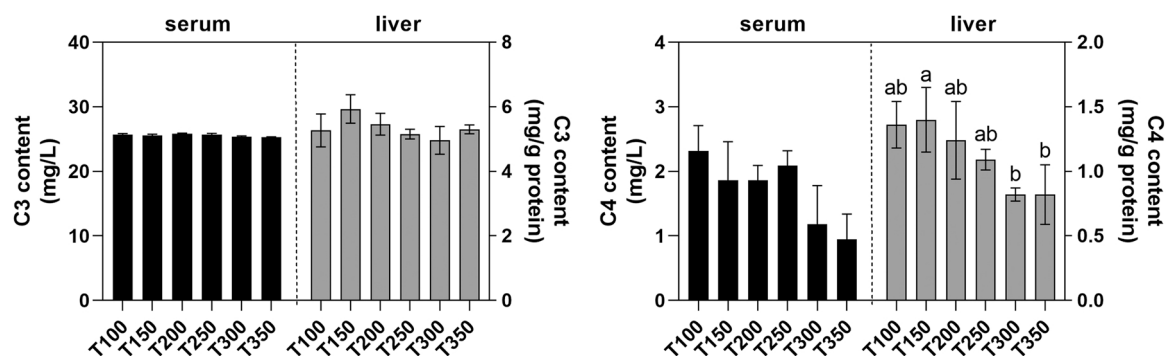


Fig. 5. Effects of crowding stress on complement C3 and C4 of hybrid sturgeon cultured at different densities (T100, T150, T200, T250, T300 and T350) for 60 days. Values are presented as mean $\pm$ SE ($n = 3$). Values in the column with different letters are significantly different ($P < 0.05$). C3: Complement component 3; C4: Complement component 4.

(feelers) that project from their lower jaw and are used to detect food (Vecsei and Peterson, 2004). Therefore, crowding stress mean that the upper barbels could not touch food; this affected growth performance in the HD group.

Ascorbic acid is an essential antioxidant and plays functional roles in other enzymatic reactions and cellular processes, such as growth and mitosis (Noctor and Foyer, 1998; Smirnov and Wheeler, 2000). Tyrosinase can convert tyrosine directly to dihydroxyphenylalanine (DOPA)

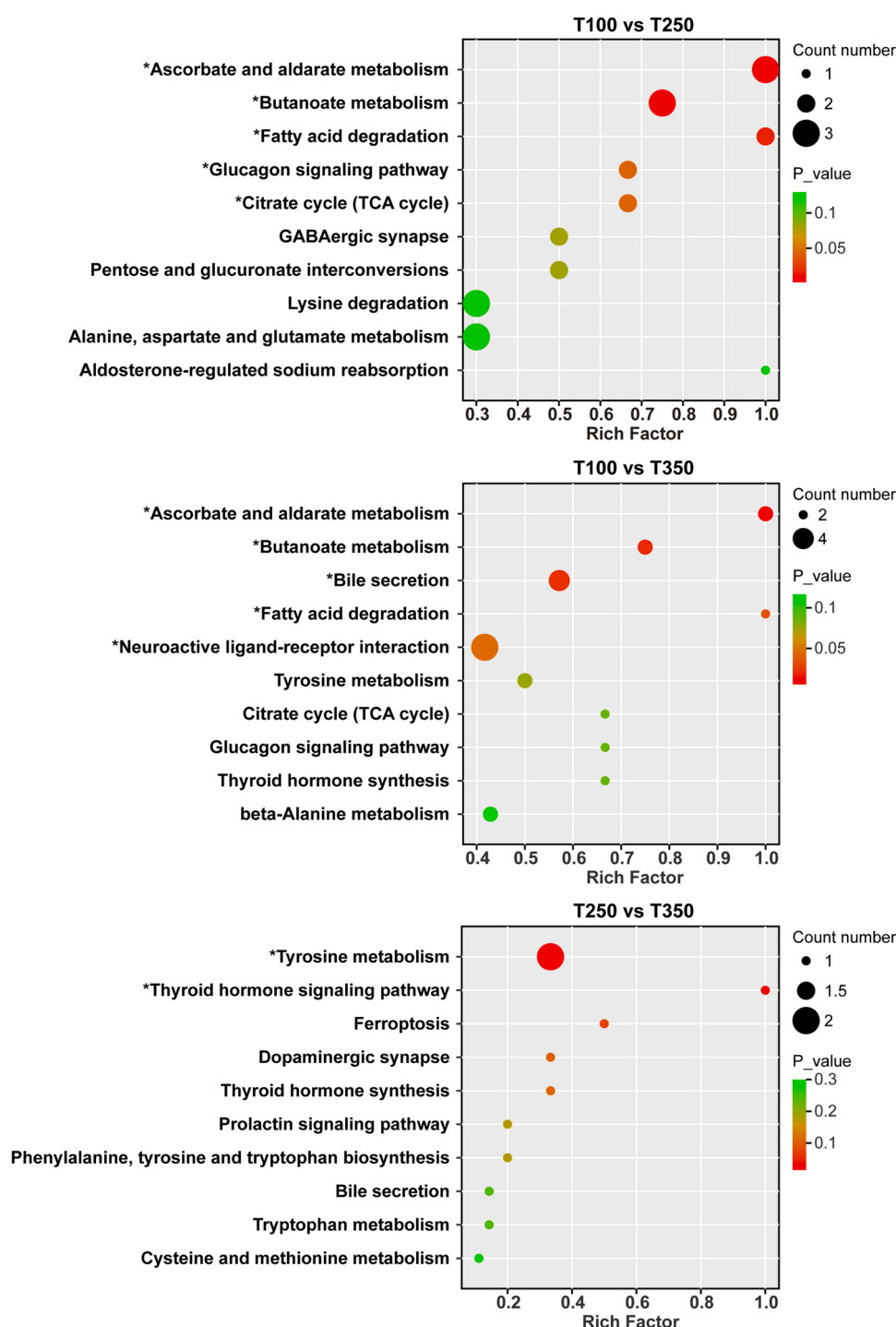


Fig. 6. KEGG pathways for differential metabolites in the serum of hybrid sturgeon. Rich factors indicate the ratio of metabolites enriched in the pathway among the metabolites annotated in the pathway. KEGG pathways with an asterisk were significantly enriched. * P value < 0.05.

in the presence of ascorbic acid (Kurpejović et al., 2021). Butanoate metabolism increases fatty acid oxidation in the muscle and adipose tissue, thus inducing lipolysis and increased energy expenditure (Henagan et al., 2015; McNabney and Henagan, 2017). Therefore, negative effects on the regulation of growth in hybrid sturgeon in the MD and HD groups involved effects on ascorbate and aldarate metabolism, butanoate metabolism and fatty acid degradation. Xylulose-5-phosphate was expressed at high levels at the highest density of T350; this activated protein phosphatase and regulated glucose metabolism and fat synthesis (Kabashima et al., 2003).

4.2. Effects of crowding stress in juvenile hybrid sturgeon

With an increase of stocking density, the serum levels of cortisol and glucose increased significantly; the levels of adrenal corticotrophic hormone (ACTH), as an upstream regulatory molecule, also increased (Angelousi et al., 2000). Cortisol is widely known as the “stress hormone”, its level was previously shown to be positively correlated with stocking density (Adineh et al., 2021; Fatima et al., 2018). In this study, the metabolite fumaric acid was also positively correlated with stocking density. Fumaric acid also involved in urea synthesis during the TCA cycle to reduce the ammonia-nitrogen stress (Natnan et al., 2021). Blood

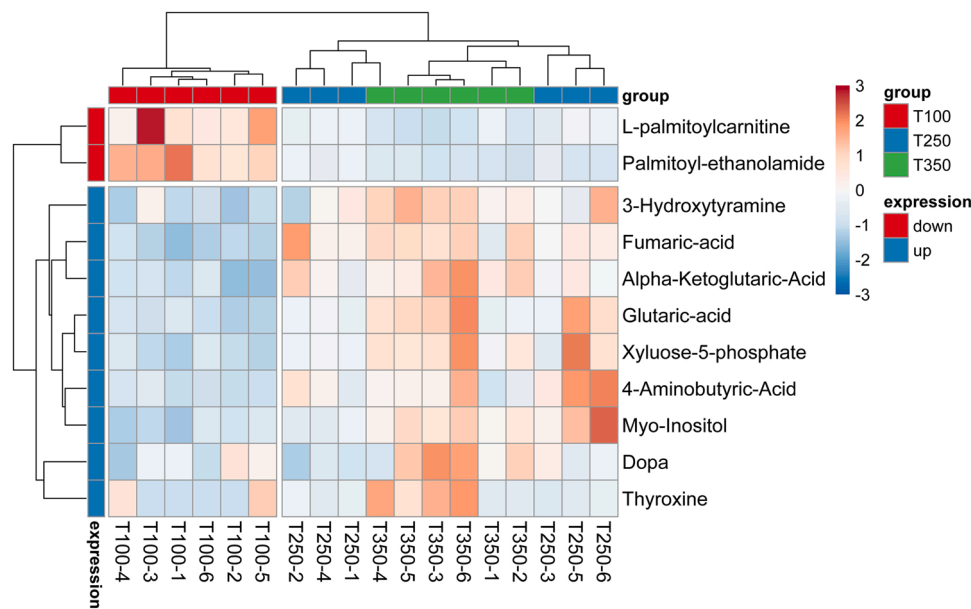


Fig. 7. Hierarchical cluster analysis of differential metabolites. Hybrid sturgeon were cultured at densities of T100, T250 and T350 for 60 days. The horizontal axis shows the sample name while the vertical axis shows the metabolites; the signal intensities of metabolites were normalized by unit variance scaling.

glucose levels generally follow the same trend as cortisol; this is thought to promote gluconeogenesis or glycogen breakdown (Khani and Tayek, 2001). We also found that the glucagon signaling pathway and citrate cycle was activated in T250; this accelerates glycogenolysis via the cAMP-PK system (La Sala and Pontiroli, 2021). The process of gluconeogenesis and glycogenolysis can help to increase the energy expenditure of fish under stress (Kleinert et al., 2019). The levels of glucose and cortisol exhibit a slight decreasing trend as crowding stress increased after the 60-day period of culture. The same trend was also observed previously under chronic crowding stress; this was considered to be an adaptive ability of fish under conditions of long-term stress (Ni, M. et al., 2014). Increased levels of 4-aminobutyric acid have been implicated as the main mechanism underlying metabolic depression during anoxia (Lardon et al., 2013). myo-inositol is a protective agent shared by all eukaryotes exposed to hypertonic stress (Boundedjah et al., 2012) and has also been observed in crowding stress, thus suggesting that 4-aminobutyric acid and myo-inositol could be used as new indicators of crowding stress.

4.3. Effects of crowding stress on the antioxidant capacity of juvenile hybrid sturgeon

Previous study showed that the activities of SOD and GSH-PX in the serum were significantly reduced under crowding stress in Chinese sturgeon (Long et al., 2019). MDA is one of the final products of polyunsaturated fatty acids peroxidation in the cells; an increase in free radicals causes overproduction of MDA (Gawel et al., 2004). Similar with our study, the antioxidant enzyme activity (SOD and CAT) were decreased but levels of MDA were increased thus indicating that crowding stress inhibited the antioxidant capacity and caused lipid peroxidation in the livers of hybrid sturgeon (Liu et al., 2014; Long et al., 2019; Yousefi et al., 2020).

Alpha-ketoglutaric acid has been shown to affect the development and maturation of offspring of American minks (*Neovison vison*) (Dobrowolski et al., 2021), which is a well-known metabolic intermediate of various processes and performs a protective role against lipid peroxidation (Velvizhi et al., 2002). Ascorbate and aldarate metabolism are known to be part of a pathway related to antioxidant defense that is able to effectively scavenge superoxide and prevent tyrosine nitration (Smirnov, 2018; Zhao et al., 2018). Crowding stress is known to alter the

pathways of ascorbate and aldarate metabolism; ascorbate cooperates with SOD to remove superoxide and reduce the formation of peroxynitrite (Smirnov, 2018). Similar with other teleost, plasma levels of GSH-PX activity did not change but the levels of GSH were significantly higher under crowding stress (Djordjevic et al., 2021; Du et al., 2019). GSH is an important antioxidant and protects cells and enzymes from oxidative stress. GSH-PX is a cytosolic enzyme that catalyzes the reduction of hydrogen peroxide to protect cells (Fanucchi, 2014). However, the antioxidant capacity of ascorbate is more reactive than GSH and represents a preferred pathway because one electron donation by GSH produces the reactive and damaging thiyl radical (Gebicki et al., 2010). Therefore, ascorbate and aldarate metabolism contributed part of the antioxidant capacity under crowding stress.

4.4. Effects of crowding stress on thyroid hormones in juvenile hybrid sturgeon

We found that tyrosine metabolism and the thyroid hormone signaling pathway were significantly enriched in the HD group. The hypothalamic-pituitary-thyroid axis regulates energy metabolism by secreting thyroid hormone to ensure the normal development and growth of fish (Blanton and Specker, 2007). The metabolism of thyroid hormones alters iodothyronine deiodinase activity and gene expression with the HPT axis; these changes may affect the complement system (Gao et al., 2020). Previous studies found that the levels of thyroxine (T4) decreased with increasing stocking density; these findings were similar to those found with *Labeo rohita* (Fatima et al., 2018) and *Puntius sarana* (Upadhyay et al., 2022), thus suggesting that the activity of the HPT axis was suppressed, possibly due to the reduced food intake in the HD group. However, a weak link between the thyroid and appetite was previously reported in fish (Deal and Volkoff, 2021). Crowding stress plays a major role in the elevation of cortisol level which exhibits negative feedback on the hypothalamus-pituitary-inter-renal axis, thus reducing the peripheral circulation of T3 and T4 (Swain et al., 2022).

4.5. Effects of crowding stress on innate immunity in juvenile hybrid sturgeon

Previous studies have proved that high stocking density acts as a stressor and causes immunosuppression in fish such as common carp,

turbot (*Scophthalmus maximus*), gilthead seabream (*Sparus aurata*), and grass carp (Adineh et al., 2021; Hoseini et al., 2019; Jia et al., 2016; Lin et al., 2018; Montero et al., 1999). In this study, the levels of several key immune parameters (C3, C4, IgM and LZM) did not change after chronic crowding stress. However, the circulating levels of IgM increased with increased water temperature and salinity (Dominguez et al., 2004), thus suggesting that the immune system can be altered by certain factors in the aquatic environment. One potential for this is that crowding stress represents a social factor involved in the stimulation of the hypothalamic-pituitary-adrenal axis (Bugajski, 1999). The number of B lymphocytes remain at normal levels under social stress and the immune system did not respond to secrete antibodies.

We also found that the levels of two metabolites, L-palmitoylcarnitine and palmitoyl-ethanolamide, were decreased after crowding stress; these metabolites are known to be involved in processes related to immune response. Palmitoyl-ethanolamide has an anti-inflammatory effect and can relieve inflammatory and neuropathic pain, especially pain induced by mechanical hyperalgesia (Seol et al., 2017). Palmitoylcarnitine is associated with the induction of both IL-6 and Ca^{2+} influx and alters the host response in the lymph nodes (Hulme et al., 2017), thus suggesting the potential use of palmitoylcarnitine profiling as a biomarker for metabolic disturbance (Al-Bakheit et al., 2016). Therefore, the levels of L-palmitoylcarnitine and palmitoyl-ethanolamide decreased after crowding stress, thus suggesting metabolic disturbance and the induction of immunosuppression in hybrid sturgeon.

5. Conclusion

High stocking density negatively influence the growth performance of juvenile hybrid sturgeon for a 60-day culture period, which mainly involved pathways associated with ascorbate and aldarate metabolism, butanoate metabolism and fatty acid degradation. The gluconeogenesis and glycogenolysis were activated by the glucagon signaling pathway in order to meet the increased energy expenditure of fish under stress. The pathways of ascorbate and aldarate metabolism were activated in MD and HD group to scavenge ROS caused by crowding stress, played a protective role against lipid peroxidation in the liver. Therefore, the ascorbate and aldarate metabolism as one of the key metabolic pathways involved in the growth, energy expenditure and antioxidant capacity of juvenile hybrid sturgeon under crowding stress.

CRediT authorship contribution statement

Conceived and designed the experiments: **Baoliang Bi** and **Yu Gao**; Performed the experiments: **Yao Zhao**; Collected the samples: **Yin Yuan**, **Yao Zhao**, **Mingyi He** and **Haoqing Song**; Analyzed the data-software: **Yao Zhao** and **Yu Gao**; Analyzed the data-visualization: **Yu Gao**; Resources and supervision: **Lingfu Kong**; **Yu Gao** wrote the manuscript with contributions from **Baoliang Bi** and **Yin Yuan**. Funding acquisition: **Baoliang Bi** and **Yu Gao**. All authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

No conflict of interest exists in the submission of this manuscript. All authors of this paper have read and approved the final version submitted. The content of this manuscript is original research that has not been published previously and is not under consideration for publication elsewhere.

Data Availability

No data was used for the research described in the article.

Acknowledgements

This work was jointly supported by the Expert Workstation of Yunnan Province of China (202005AF150042); the Key Project of Yunnan Natural Science Foundation (202202AE090018); Natural Science Foundation of Yunnan Province of China (202101BD070001-119) and the National Natural Science Foundation of China (32002360).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.aqrep.2023.101468.

References

- Adineh, H., Naderi, M., Yousefi, M., Khademi Hamidi, M., Ahmadi, E., Hoseini, S.M., 2021. Dietary licorice (*Glycyrrhiza glabra*) improves growth, lipid metabolism, antioxidant and immune responses, and resistance to crowding stress in common carp, *Cyprinus carpio*. *Aquac. Nutr.* 27 (2), 417–426. <https://doi.org/10.1111/anu.13194>.
- Al-Bakheit, A., Traka, M., Saha, S., Mithen, R., Melchini, A., 2016. Accumulation of palmitoylcarnitine and its effect on pro-inflammatory pathways and calcium influx in Prostate Cancer. *Prostate* 76 (14), 1326–1337. <https://doi.org/10.1002/pros.23222>.
- Angelousi, A., Margioris, A.N., Tsatsanis, C., Feingold, K.R., Anawalt, B., Boyce, A., Chrousos, G., Herder, W.W., Dhatriya, K., Dungan, K., Hershman, J.M., Hofland, J., Kalra, S., Katsas, G., Koch, C., Kopp, P., Korbonits, M., Kovacs, C.S., Kuohung, W., Laferriere, B., Levy, M., McGee, E.A., McLachlan, R., Morley, J.E., 2000. New. In: Purnell, J., Sahay, R., Singer, F., Sperling, M.A., Stratakis, C.A., Trencle, D.L., Wilson, D.P. (Eds.), *ACTH Action on the Adrenals*. Endotext. South, Dartmouth (MA): MDText.com.
- Blanton, M.L., Specker, J.L., 2007. The hypothalamic-pituitary-thyroid (HPT) axis in fish and its role in fish development and reproduction. *Crit. Rev. Toxicol.* 37 (1–2), 97–115. <https://doi.org/10.1080/10408440601123529>.
- Boundedjah, O., Hamon, L., Savarin, P., Desforages, B., Curmi, P.A., Pastre, D., 2012. Macromolecular crowding regulates assembly of mRNA stress granules after osmotic stress: new role for compatible osmolytes. *J. Biol. Chem.* 287 (4), 2446–2458. <https://doi.org/10.1074/jbc.M111.292748>.
- Bugajski, J., 1999. Social stress adapts signalling pathways involved in stimulation of the hypothalamic-pituitary-adrenal axis. *J. Physiol. Pharmacol.* 50 (3), 367–379.
- Çelikkale, M.S., Memiş, D., Ercan, E., Çağiltay, F., 2005. Growth performance of juvenile Russian sturgeon (*Acipenser gueldenstaedtii* Brandt & Ratzenburg, 1833) at two stocking densities in net cages. *J. Appl. Ichthyol.* 21 (1), 14–18. <https://doi.org/10.1111/j.1439-0426.2004.00567.x>.
- Deal, C.K., Volkoff, H., 2021. Effects of thyroxine and propylthiouracil on feeding behavior and the expression of hypothalamic appetite-regulating peptides and thyroid function in goldfish (*Carassius auratus*). *Peptides* 142, 170578. <https://doi.org/10.1016/j.peptides.2021.170578>.
- Dediu, L., Docan, A., Crețu, M., Grecu, I., Mogodan, A., Maereanu, M., Oprea, L., 2021. Effects of stocking density on growth performance and stress responses of bester and bester ♀ × beluga ♂ juveniles in recirculating aquaculture systems. *Animals* 11 (8), 2292. <https://doi.org/10.3390/ani11082292>.
- Djordjevic, B., Morales-Lange, B., McLean Press, C., Olson, J., Lagos, L., Mercado, L., Overland, M., 2021. Comparison of circulating markers and mucosal immune parameters from skin and distal intestine of atlantic salmon in two models of acute stress. *Int. J. Mol. Sci.* 22 (3), 1028. <https://doi.org/10.3390/ijms22031028>.
- Dobrowolski, P., Muszyński, S., Donaldson, J., Jakubczak, A., Żmuda, A., Taszkun, I., Rycerz, K., Mielnik-Błaszczak, M., Kuc, D., Tomaszewska, E., 2021. The Effects of Prenatal Supplementation with β-Hydroxy-β-Methylbutyrate and/or Alpha-Ketoglutaric Acid on the Development and Maturation of Mink Intestines Are Dependent on the Number of Pregnancies and the Sex of the Offspring. *Animals* 11 (5), 1468.
- Dominguez, M., Takemura, A., Tsuchiya, M., Nakamura, S., 2004. Impact of different environmental factors on the circulating immunoglobulin levels in the Nile tilapia, *Oreochromis niloticus*. *Aquaculture* 241 (1), 491–500. <https://doi.org/10.1016/j.aquaculture.2004.06.027>.
- Du, F., Li, Y., Tang, Y., Su, S., Yu, J., Yu, F., Li, J., Li, H., Wang, M., Xu, P., 2019. Response of the gut microbiome of *Megalobrama amblycephala* to crowding stress. *Aquaculture* 500, 586–596. <https://doi.org/10.1016/j.aquaculture.2018.10.067>.
- Fanucci, M.V., 2014. Chapter 11 - development of antioxidant and xenobiotic metabolizing enzyme systems. In: Harding, R., Pinkerton, K.E. (Eds.), *The Lung*. Second edition. Academic Press, Boston, pp. 223–231.
- Fatima, S., Izhar, S., Usman, Z., Rashid, F., Kanwal, Z., Jabeen, G., Latif, A., 2018. Effects of high stocking density on condition factor and profile of free thyroxine and cortisol in *Catla catla* (Hamilton, 1822) and *Labeo rohita* (Hamilton, 1822). *Turkish J. Fish. Aquat. Sci. TURK J FISH AQUAT SC* 18 (1), 217–221.
- Gao, Y., Liu, Z., Jia, D., Hu, Q., Li, L., Tang, R., Li, D., 2020. Acute microcystin-LR exposure interfere thyroid hormones homeostasis in adult zebrafish (*Danio rerio*). *Chemosphere* 243, 125258. <https://doi.org/10.1016/j.chemosphere.2019.125258>.
- Garcia, F., Romera, D.M., Gozi, K.S., Onaka, E.M., Fonseca, F.S., Schalch, S.H.C., Candeira, P.G., Guerra, L.O.M., Carmo, F.J., Carneiro, D.J., Martins, M.I.E.G., Portella, M.C., 2013. Stocking density of Nile tilapia in cages placed in a

- hydroelectric reservoir. *Aquaculture* 410–411, 51–56. <https://doi.org/10.1016/j.aquaculture.2013.06.010>.
- Gawel, S., Wardas, M., Niedworok, E., Wardas, P., 2004. [Malondialdehyde (MDA) as a lipid peroxidation marker]. *Wiad. Lek.* 57 (9–10), 453–455.
- Gebicki, J.M., Nauser, T., Domazou, A., Steinmann, D., Bounds, P.L., Koppenol, W.H., 2010. Reduction of protein radicals by GSH and ascorbate: potential biological significance. *Amino Acids* 39 (5), 1131–1137. <https://doi.org/10.1007/s00726-010-0610-7>.
- Hao, Q., Teame, T., Wu, X., Ding, Q., Ran, C., Yang, Y., Xing, Y., Zhang, Z., Zhou, Z., 2021. Influence of diet shift from bloodworm to formulated feed on growth performance, gut microbiota structure and function in early juvenile stages of hybrid sturgeon (*Acipenser baeri* × *Acipenser schrenckii*). *Aquaculture* 533, 736165. <https://doi.org/10.1016/j.aquaculture.2020.736165>.
- Henagan, T.M., Stefanska, B., Fang, Z., Navard, A.M., Ye, J., Lenard, N.R., Devarshi, P.P., 2015. Sodium butyrate epigenetically modulates high-fat diet-induced skeletal muscle mitochondrial adaptation, obesity and insulin resistance through nucleosome positioning. *Br. J. Pharmacol.* 172 (11), 2782–2798. <https://doi.org/10.1111/bph.13058>.
- Hoseini, S.M., Abtahi, B., Yousefi, M., 2019. Effects of fasting on metabolic and immunological responses of common carp (*Cyprinus carpio*) to a further acute stress. *Aquac. Res.* 50 (4), 1177–1185. <https://doi.org/10.1111/are.13992>.
- Hulme, H.E., Meikle, L.M., Wessel, H., Strittmatter, N., Swales, J., Thomson, C., Nilsson, A., Nibbs, R.J.B., Milling, S., Andren, P.E., Mackay, C.L., Dexter, A., Bunch, J., Goodwin, R.J.A., Burchmore, R., Wall, D.M., 2017. Mass spectrometry imaging identifies palmitoylcarnitine as an immunological mediator during *Salmonella* Typhimurium infection. *Sci. Rep.* -Uk 7 (1), 2786. <https://doi.org/10.1038/s41598-017-03100-5>.
- Jia, R., Liu, B.-L., Feng, W.-R., Han, C., Huang, B., Lei, J.-L., 2016. Stress and immune responses in skin of turbot (*Scophthalmus maximus*) under different stocking densities. *Fish. Shellfish Immunol.* 55, 131–139. <https://doi.org/10.1016/j.fsi.2016.05.032>.
- Kabashima, T., Kawaguchi, T., Wadzinski, B.E., Uyeda, K., 2003. Xylulose 5-phosphate mediates glucose-induced lipogenesis by xylulose 5-phosphate-activated protein phosphatase in rat. *Liver* 100 (9), 5107–5112. <https://doi.org/10.1073/pnas.0730817100>.
- Karnatak, G., Das, B.K., Mishal, P., Tayung, T., Kumari, S., Sarkar, U.K., Das, A.K., Ali, Y., 2021. Impact of stocking density on growth, feed utilization and survival of cage reared minor carp, Labeo bata (Hamilton, 1822) in Maithon reservoir, India. *Aquaculture* 532, 736078. <https://doi.org/10.1016/j.aquaculture.2020.736078>.
- Khani, S., Tayek, J.A., 2001. Cortisol increases gluconeogenesis in humans: its role in the metabolic syndrome. *Clin. Sci. (Lond.)* 101 (6), 739–747. <https://doi.org/10.1042/cs1010739>.
- Kleinert, M., Sachs, S., Habegger, K.M., Hofmann, S.M., Muller, T.D., 2019. Glucagon regulation of energy expenditure. *Int. J. Mol. Sci.* 20 (21) <https://doi.org/10.3390/ijms20215407>.
- Kurpejović, E., Wendisch, V.F., Sariyar Akbulut, B., 2021. Tyrosinase-based production of l-DOPA by *Corynebacterium glutamicum*. *Appl. Microbiol. Biotechnol.* 105 (24), 9103–9111. <https://doi.org/10.1007/s00253-021-11681-5>.
- La Sala, L., Pontiroli, A.E., 2021. New Fast Acting Glucagon for Recovery from Hypoglycemia, a Life-Threatening Situation: Nasal Powder and Injected Stable Solutions. *Int. J. Mol. Sci.* 22 (19), 10643.
- Lardon, I., Nilsson, G.E., Stecyk, J.A.W., Vu, T.N., Laukens, K., Dommissie, R., De Boeck, G., 2013. ¹H-NMR study of the metabolome of an exceptionally anoxia tolerant vertebrate, the crucian carp (*Carassius carassius*). *Metabolomics* 9 (2), 311–323. <https://doi.org/10.1007/s11306-012-0448-y>.
- Lin, W., Li, L., Chen, J., Li, D., Hou, J., Guo, H., Shen, J., 2018. Long-term crowding stress causes compromised nonspecific immunity and increases apoptosis of spleen in grass carp (*Ctenopharyngodon idella*). *Fish. Shellfish Immunol.* 80, 540–545. <https://doi.org/10.1016/j.fsi.2018.06.050>.
- Liu, B., Xu, P., Xie, J., Ge, X., Xia, S., Song, C., Zhou, Q., Miao, L., Ren, M., Pan, L., Chen, R., 2014. Effects of emodin and vitamin E on the growth and crowding stress of Wuchang bream (*Megalobrama amblycephala*). *Fish. Shellfish Immunol.* 40 (2), 595–602. <https://doi.org/10.1016/j.fsi.2014.08.009>.
- Long, L., Zhang, H., Ni, Q., Liu, H., Wu, F., Wang, X., 2019. Effects of stocking density on growth, stress, and immune responses of juvenile Chinese sturgeon (*Acipenser sinensis*) in a recirculating aquaculture system. *Comp. Biochem. Physiol. C. Toxicol. Pharm.* 219, 25–34. <https://doi.org/10.1016/j.cbpc.2019.02.002>.
- McNabney, S.M., Henagan, T.M., 2017. Short chain fatty acids in the colon and peripheral tissues: a focus on butyrate, colon cancer, obesity and insulin resistance. *Nutrients* 9 (12), 1348.
- Montero, D., Marrero, M., Izquierdo, M.S., Robaina, L., Vergara, J.M., Tort, L., 1999. Effect of vitamin E and C dietary supplementation on some immune parameters of gilthead seabream (*Sparus aurata*) juveniles subjected to crowding stress. *Aquaculture* 171 (3), 269–278. [https://doi.org/10.1016/S0044-8486\(98\)00387-1](https://doi.org/10.1016/S0044-8486(98)00387-1).
- Nakao, M., Tsujikura, M., Ichiki, S., Vo, T.K., Somamoto, T., 2011. The complement system in teleost fish: progress of post-homolog-hunting researches. *Dev. Comp. Immunol.* 35 (12), 1296–1308. <https://doi.org/10.1016/j.dci.2011.03.003>.
- Natnan, M.E., Low, C.-F., Chong, C.-M., Bunawan, H., Baharum, S.M., 2021. Integration of Omics Tools for Understanding the Fish Immune Response Due to Microbial Challenge. *Front. Mar. Sci.* 8, 668711 <https://doi.org/10.3389/fmars.2021.668777>.
- Ni, M., Wen, H., Li, J., Chi, M., Bu, Y., Ren, Y., Zhang, M., Song, Z., Ding, H., 2014. The physiological performance and immune responses of juvenile Amur sturgeon (*Acipenser schrenckii*) to stocking density and hypoxia stress. *Fish. Shellfish Immunol.* 36 (2), 325–335. <https://doi.org/10.1016/j.fsi.2013.12.002>.
- Ni, M., Wen, H., Li, J., Chi, M., Bu, Y., Ren, Y., Zhang, M., Song, Z., Ding, H., 2014. The physiological performance and immune responses of juvenile Amur sturgeon (*Acipenser schrenckii*) to stocking density and hypoxia stress. *Fish. Shellfish Immunol.* 36 (2), 325–335. <https://doi.org/10.1016/j.fsi.2013.12.002>.
- Ni, M., Wen, H., Li, J., Chi, M., Bu, Y., Ren, Y., Zhang, M., Song, Z., Ding, H., 2016. Effects of stocking density on mortality, growth and physiology of juvenile Amur sturgeon (*Acipenser schrenckii*). *Aquac. Res.* 47 (5), 1596–1604. <https://doi.org/10.1111/are.12620>.
- Noctor, G., Foyer, C.H., 1998. Ascorbate and glutathione: keeping active oxygen under control. *Annu. Rev. Plant Biol.* 49 (1), 249–279. <https://doi.org/10.1146/annurev.arplant.49.1.249>.
- Ohkawa, H., Ohishi, N., Yagi, K., 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal. Biochem.* 95 (2), 351–358. [https://doi.org/10.1016/0003-2697\(79\)90738-3](https://doi.org/10.1016/0003-2697(79)90738-3).
- Ortuño, J., Esteban, M.A., Meseguer, J., 2001. Effects of short-term crowding stress on the gilthead seabream (*Sparus aurata* L.) innate immune response. *Fish. Shellfish Immunol.* 11 (2), 187–197. <https://doi.org/10.1006/fsim.2000.0304>.
- Ramsay, J.M., Feist, G.W., Varga, Z.M., Westerfield, M., Kent, M.L., Schreck, C.B., 2006. Whole-body cortisol is an indicator of crowding stress in adult zebrafish, *Danio rerio*. *Aquaculture* 258 (1), 565–574. <https://doi.org/10.1016/j.aquaculture.2006.04.020>.
- Ray, S.N.C., Sinha, R.C., 2014. Serum Cortisol and Glucose: Reliable Bioindicators of stress in the Fish *Labeo rohita*. *J. Innov.* 1, 6–17.
- Regoli, F., Gorbi, S., Frenzilli, G., Nigro, M., Corsi, I., Focardi, S., Winston, G.W., 2002. Oxidative stress in ecotoxicology: from the analysis of individual antioxidants to a more integrated approach. *Mar. Environ. Res.* 54 (3–5), 419–423. [https://doi.org/10.1016/s0141-1136\(02\)00146-0](https://doi.org/10.1016/s0141-1136(02)00146-0).
- Saurabh, S., Sahoo, P.K., 2008. Lysozyme: an important defence molecule of fish innate immune system. *Aquac. Res.* 39 (3), 223–239. <https://doi.org/10.1111/j.1365-2109.2007.01883.x>.
- Seol, T.-K., Lee, W., Park, S., Kim, K.N., Kim, T.Y., Oh, Y.N., Jun, J.H., 2017. Effect of palmitoylethanolamide on inflammatory and neuropathic pain in rats. *Korean J. Anesth.* 70 (5), 561–566. <https://doi.org/10.4097/kjae.2017.70.5.561>.
- Smirnoff, N., 2018. Ascorbic acid metabolism and functions: a comparison of plants and mammals. *Free Radic. Biol. Med.* 122, 116–129. <https://doi.org/10.1016/j.freeradbiomed.2018.03.033>.
- Smirnoff, N., Wheeler, G.L., 2000. Ascorbic acid in plants: biosynthesis and function. *Crit. Rev. Biochem. Mol. Biol.* 35 (4), 291–314. <https://doi.org/10.1080/10409230008984166>.
- Swain, H.S., Das, B.K., Upadhyay, A., Ramteke, M.H., Kumar, V., Meena, D.K., Sarkar, U. K., Chadha, N.K., Rawat, K.D., 2022. Stocking density mediated stress modulates growth attributes in cage reared *Labeo rohita* (Hamilton) using multifarious biomarker approach. *Sci. Rep.* -Uk 12 (1), 9869. <https://doi.org/10.1038/s41598-022-13570-x>.
- Taylor, J., King, R.D., Altmann, T., Fiehn, O., 2002. Application of metabolomics to plant genotype discrimination using statistics and machine learning. *Bioinformatics* 18 (suppl_2), S241–S248. doi: https://doi.org/10.1093/bioinformatics/18.suppl_2.S241.
- Upadhyay, A., Swain, H.S., Das, B.K., Ramteke, M.H., Kumar, V., Krishna, G., Mohanty, B.P., Chadha, N.K., Das, A.K., 2022. Stocking density matters in open water cage culture: Influence on growth, digestive enzymes, haemato-immuno and stress responses of *Puntius sarana* (Ham, 1822). *Aquaculture* 547, 737445. <https://doi.org/10.1016/j.aquaculture.2021.737445>.
- Vecsei, P., Peterson, D., 2004. Sturgeon ecomorphology: a descriptive approach. In: LeBreton, G.T.O., Beamish, F.W.H., McKinley, R.S. (Eds.), *Sturgeons and Paddlefish of North America*. Springer, Netherlands, Dordrecht, pp. 103–133.
- Velizhi, S., Dakshayani, K.B., Subramanian, P., 2002. Effects of α-ketoglutarate on antioxidants and lipid peroxidation products in rats treated with ammonium acetate. *Nutrition* 18 (9), 747–750. [https://doi.org/10.1016/S0899-9007\(02\)00825-0](https://doi.org/10.1016/S0899-9007(02)00825-0).
- Wafa, A.H., Pierre, D., Daniel, B., 2004. Modelling growth and food intake rhythms of brook trout *Salvelinus fontinalis* under the effects of density and ration. *Ecol. Model.* 175 (4), 385–394. <https://doi.org/10.1016/j.ecolmodel.2003.10.024>.
- Wunderink, Y.S., Engels, S., Halm, S., Yufera, M., Martinez-Rodriguez, G., Flik, G., Klaren, P.H., Mancera, J.M., 2011. Chronic and acute stress responses in Senegalese sole (*Solea senegalensis*): the involvement of cortisol, CRH and CRH-BP. *Gen. Comp. Endocrinol.* 171 (2), 203–210. <https://doi.org/10.1016/j.ygcen.2011.01.010>.
- Yang, D.G., Zhu, Y.J., Luo, Y.P., Zhao, J.H., Chen, J.W., 2011. Effect of stocking density on growth performance of juvenile Amur Sturgeon (*Acipenser schrenckii*). *J. Appl. Ichthyol.* 27 (2), 541–544. <https://doi.org/10.1111/j.1439-0426.2011.01705.x>.
- Yousefi, M., Shabunin, S.V., Vatikov, Y.A., Kulikov, E.V., Adineh, H., Khademi Hamidi, M., Hoseini, S.M., 2020. Effects of lavender (*Lavandula angustifolia*) extract inclusion in diet on growth performance, innate immunity, immune-related gene expression, and stress response of common carp, *Cyprinus carpio*. *Aquaculture* 515, 734588. <https://doi.org/10.1016/j.aquaculture.2019.734588>.
- Zhao, L., Huang, Y., Paglia, K., Vaniya, A., Wancewicz, B., Keller, A.A., 2018. Metabolomics reveals the molecular mechanisms of copper induced cucumber leaf (*Cucumis sativus*) senescence. *Environ. Sci. Technol.* 52 (12), 7092–7100. <https://doi.org/10.1021/acs.est.8b00742>.