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Effects of long-term crowding stress on neuro-endocrine-immune network of rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

Low levels of stresses cause eustress while high stressful situations result in distress. Female rainbow trout (*Oncorhynchus mykiss*) was reared under crowded conditions to mimic the stressful environment of intensive fishery production. Trout was stocked for 300 days with initial densities of 4.6 ± 0.02 (final: 31.1 ± 0.62), 6.6 ± 0.03 (final: 40.6 ± 0.77), and 8.6 ± 0.04 (final: 49.3 ± 1.09) kg/m^3 as SD1, SD2 and SD3. We assessed molecular, cellular and organismal parameters to understand the flexibility of neuro-endocrine-immune network during stress. Trout with higher initial density (SD3) displayed the slightly activated hypothalamus-pituitary-interrenal (HPI) axis with positively increased antioxidant enzyme activities and anti-inflammatory cytokine transcriptions on day 60 or 120. These results indicated that low level of stress was capable of exerting eustress by activating neuro-endocrine-immune network with beneficial adaptation. Transition from eustress to distress was induced by the increased intensity and duration of crowding stress on day 240 and 300. The prolonged activation of HPI axis resulted in suppressed growth hormone-insulin-like growth factor (GH-IGF) axis, up-regulated cytokine transcriptions and severe reactive oxygen species stress. Stress means reset of neuro-endocrine-immune network with energy expenditure and redistribution. Digestive ability of trout with distress was also inhibited on day 240 and 300, indicating a decreased total energy supplement and energy distribution for functions are not necessary for surviving such as growth and reproduction. Consequently, we observed the dyshomeostasis of energy balance and neuro-endocrine-immune network of trout during long-term crowding conditions.

1. Introduction

With the introduction of intensive agriculture (aquaculture), larger food production is possible with less amount of land (water), providing affordable, safe, and environment-friendly foods, which may further aid in solving the worldwide hunger problems. However, animals (fishes) often suffer confinement as they are artificially reared in a density that is much higher than they would experience in wild conditions. There is a continuous discussion of impaired animal welfare and increased environment risk due to confinement at high density [1,2]. For example, infectious diseases in livestock can be exacerbated by high density and further transmitted to wildlife animals in the nearby natural environment, resulting in increased risk to animal health, environment health and human health [3,4].

In the aquatic environment, high density is widely recognized as a stressor that may affect animal welfare and animal harvest [5,6]. Effects of high density on growth, physiology, health and welfare of several teleost species have been reported [7–12]. Conflicting results of growth and feeding performance in high density are reported in three important species: seabream (*Sparus aurata*) shows no reduction [8] while rainbow trout (*Oncorhynchus mykiss*) and sea bass (*Dicentrarchus labrax*) show significant decrease [13,14]. Furthermore, a decrease in appetite leads to accumulation of uneaten feed which may result in poor environment water quality, potentially affecting other organisms in nearby water environment. In physiology, high density causes significantly increased plasma cortisol and metabolic cost [15]. Reduced immunocompetence is also observed in fish at inappropriate density [8,11,16,17], increasing the susceptibility to pathogens and opportunities for pathogens outbreak among the environment [18]. Crowded conditions also alter hematological and immunological parameters [19,20]. Interestingly, a 9-month study shows that both low and high densities have the potential to adversely affect trout welfare [10].

Day's definition of stress is the body's multi-system responses to challenges that may overwhelm the homeostatically responsive mechanisms [21]. Schreck and Tort identified that “the general physiological response of fish to threatening situations, as with all vertebrates, is

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Abbreviations

ACTH (*acth*) Adrenocorticotropic hormone
CAT Catalase
CRH (*crh*) Corticotropin-releasing hormone
GAS General adaptation syndrome
GPx Glutathione peroxidase
GH (*gh*) Growth hormone
HPI Hypothalamus–pituitary–interrenal
IGF-1 (*igf-1*) Insulin-like growth factor 1
IL-1β/IL-10 (*il-1β/il-10*) interleukin 1 beta/interleukin 10
ROS Reactive oxygen species
SOD Superoxide dismutase
SD1, 2, 3 Stocking density 1, 2 and 3
TNF-α (*tnf-α*) Tumor necrosis factor alpha
TGF-β1 (*tgf-β1*) Transforming growth factor beta 1

referred to as stress [22]. Previous studies revealed that stress can disturb dynamic balance between maintenance energy (to maintain basal metabolism and cope with stress) and production energy (to regulate growth, reproduction and immunity) [23]. Adaptive (or resistant) progress overcomes the disturbance of environmental stress [24,25] and reshapes allostasis by selectively stimulating or inhibiting the energy utilization of certain physiological functions. Adaptive (or resistant) progress may enable fish to fully or sufficiently overcome the stressors, restoring homeostasis totally or partially. Otherwise, fish would start a trajectory to death [22].

General adaptation syndrome is proposed by Selye in 1950 as a hormonal cascade that produces all other responses to stressors [26]. Exposure to stressors results in the activation of the hypothalamus-pituitary-interrenal (HPI) axis in fishes [27], causing the release of glucocorticoids, such as cortisol [28,29]. The HPI axis shows crossover with other non-stress axes as the hypothalamus integrates control over the multiple functions including stress handing, energy redistribution, immunity resistances and growth performance [30,31]. For example, prolonged HPI activation may spare the energy that is not necessary to survive [32], suppressing other endocrine axes. We also have confirmed that high density may suppress the female reproductive functions of trout [33]. Ovarian development could require a larger amount of energy when compared to the development of testis, thus the set points of growth performance and endocrine homeostasis in females may be dramatically altered by ovary development and the prolonged activation of HPI axis.

Several studies reported that high density disrupts physiology and behavior of fish [14,34,35]. The majority of previous studies have transferred the fish to (extremely) high density directly from low density for hours/days to mimic acute stress, or weeks/months to mimic chronic stress. However, it is difficult to define the terms of “acute” and “chronic” as they are context-dependent [22]. Directly transferring the fish from low density to high density may immediately cause stress regardless of the duration of experiment. At minimum, it would be expected that the fish would initially suffer from very high stress levels, nomatter if the experimental crowding conditions are long or short term.

In this study, we elected to expose the fish to a mildly increased density, rather than a highly increased density, as this more closely correlates to the conditions of a wild aquatic environment. The initial density was low, and the intensity of crowding stress increased gradually as the trout grew. Female rainbow trout (*Oncorhynchus mykiss*) was reared for 300 days to mimic stressful environment of intensive fishery production or water loss from lakes [36]. We evaluated the endocrinal profiles and immune responses of trout in the long-term stressful conditions.

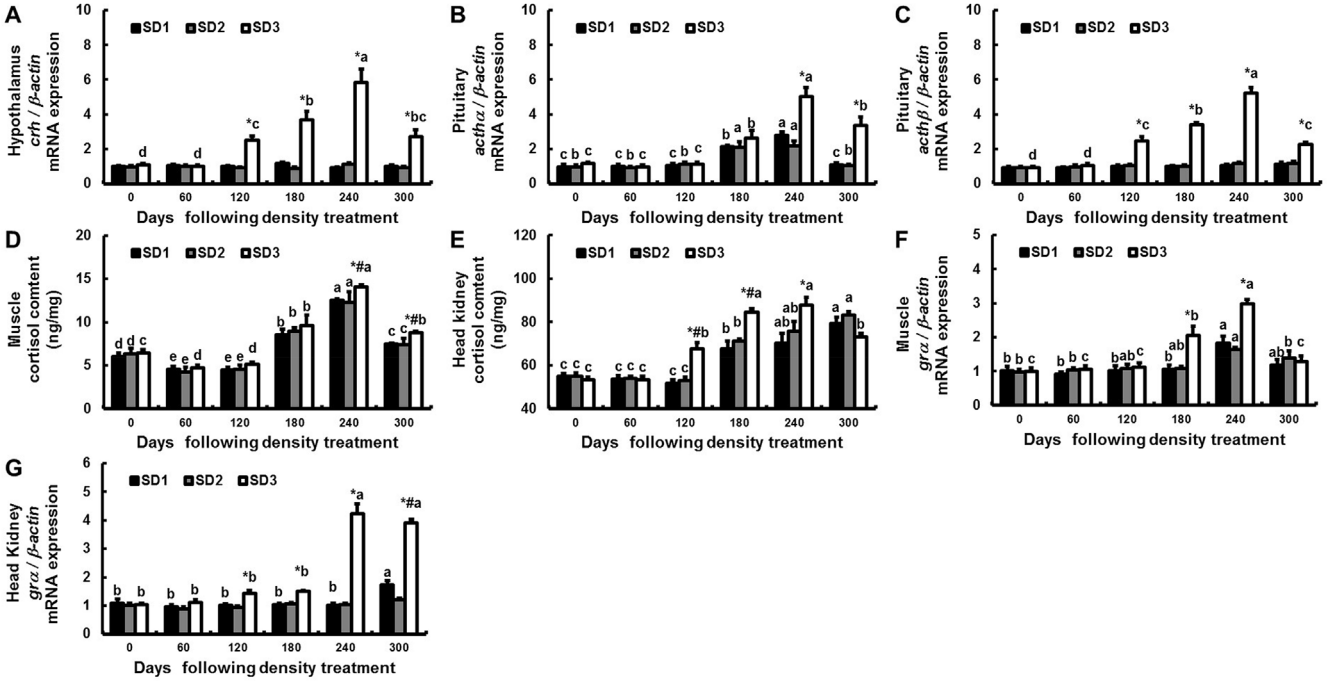

Fig. 1. HPI axis of trout in different densities. Hypothalamic *crh* (A), pituitary *actha* (B), pituitary *acthβ* (C), muscle cortisol (D), head kidney cortisol (E), muscle *grα* (F), and head kidney *grα* (G). Different letters within SD1, SD2 or SD3 indicate significant differences within each density group over all time points, * indicates significant differences between SD1 and SD2 or between SD1 and SD3 at a given time point, # indicates significant differences between SD2 and SD3 at a given time point ($P < 0.05$, two-way ANOVA, followed by Tukey's Multiple Range test). The *p*-value of density, time and interaction computed for two-way ANOVA is shown in Table 1. Data are presented as mean ± SEM of 4 pools (n = 4), except cortisol with 6 pools (n = 6) with three trout per pool each (see 2.2. for details). Fig.2–6 have same protocol of the pooled samples as Fig. 1.

2. Material and methods

2.1. Ethics statement

All experiments were conducted in conformity to the Guidelines of Animal Research and Ethics Committees of Ocean University of China (Permit Number: 20141201) as well as the guidelines of National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978). No endangered or protected species were involved in our studies.

2.2. Experimental design

Trout (~114 g) were distributed into cages (3 m × 3 m × 3 m) in three densities for 300 days as SD1 (40 fishes/m^3, 4.6 ± 0.02 kg/m^3, 1080 fishes/cage initially; 40 fishes/m^3, 31.1 ± 0.62 kg/m^3, 1080 fishes/cage finally), SD2 (60 fishes/m^3, 6.6 ± 0.03 kg/m^3, 1620 fishes/cage initially, and 60 fishes/m^3, 40.6 ± 0.77 kg/m^3, 1620 fishes/cage finally) and SD3 (80 fishes/m^3, 8.8 ± 0.04 kg/m^3, 2160 fishes/cage initially, and 80 fishes/m^3, 49.3 ± 1.09 kg/m^3, 2160 fishes/cage finally). Density variation is shown in Supplementary Table S1. Four replicate cages were set for each stocking density with same density of SD1, SD2 or SD3 during experiment (three groups for parallel sampling and one group as the supplemental group for each density treatment). Mortality was checked during feeding every day. The dead trout was replaced by the trout with similar body size from counterpart supplementary group. The mortalities during 300-day experiment were 2.21 ± 0.21% (SD1), 2.48 ± 0.24% (SD2) and 3.35% ± 0.14% (SD3). When the trout from supplementary group was moved into experimental group, same numbers of trout were also added to supplementary group, maintaining the same density between experimental and supplementary groups. Trout in supplemental and experimental groups had similar genetic background and age (from the same batch of fertilized eggs of broodstock), similarly initial body size, and similar culture (feeding) strategy. According to standards of National Excellent Rainbow Trout Seed Station of China (National Excellent Rainbow Trout Seed Station of China, Gansu Province, China), UK Farm Animal Welfare Council (FAWC), and previous studies, 40 fish/m^3 (around 4.6 kg/m^3) and 80 fish/m^3 (around 8.6 kg/m^3) were considered low and high initial stocking densities in terms of body size, and 30 kg/m^3 was considered as a relatively high density, in which trout welfare is impaired based on appearance, physiology and mortality rate [10,37,38].

Trout were fed on a commercial pellet diet with protein (40%), fat (26%), carbohydrate (14%) twice each day (8:00 a.m. and 4:00 p.m.). The feeding strategy depended on the body weight and eating responses of trout. Feeding rate was estimated by 1.5% of the body weight (evaluated) and adjusted by eating responses. Since it is not feasible to collect uneaten pellets in the reservoir, feeding was stopped when trout was eating sluggishly. Trout were starved 24 h prior to sampling. At each sampling point, all trout were individually sampled under anesthesia (MS-222, tricaine methane sulphonate, 35–45 mg/L). Rainbow trout were removed from each of the three densities on day 0, 60, 120, 180, 240, and 300 over the course of 300 days. At least six female samples were selected to record body weight (± 0.01 g). To reduce the individual variation and the experimental cost, samples from three individuals were pooled in one sample (details are shown in figure legend of Fig. 1). For "n" = 4, it means that three trout from cage 1 were pooled as sample 1. Same method was used to collect trout from cage 2 and 3 as sample 2 and sample 3. For the last sample, one trout was collected from cage 1, 2, 3, respectively, as sample 4. For "n" = 6, it means that six trout were collected from cage 1 and mixed first three as sample 1 and mixed the second three as sample 2. Likewise, same method was used to collect six trout from cage 2 as sample 3 and 4; and to collect six trout from cage 3 as sample 5 and 6. Similar body-sized trout from supplemental groups was added in each density to maintain density after sampling.

2.3. Activities of digestive and antioxidant enzymes

Gastrointestinal samples were rinsed with cold distilled water and then homogenized in 10-fold dilution (1 g per 10 ml) PBS at 4 °C. Supernatant was collected after centrifugation and stored at 4 °C. All protocols and details for the measurement of trypsin, lipase and amylase activities were described previously [39]. Muscle and head kidney samples were also homogenized and collected as the protocol of the gastrointestinal samples. Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) activities were measured via commercially colorimetric assay kit (Nanjing Jiancheng Bioengineering Institute, China). These commercial kits are validated by recently study [40].

2.4. Hormone concentration and real-time PCR analysis

Cortisol was determined by radioimmunoassay. Muscle glucose were determined with a Mindray Automated Biochemistry Analyzer [41]. Protocols of muscle homogenate, radioimmunoassay and biochemical analysis were described in our previous paper [42,43].

Total RNA of collected samples were extracted by RNAiso reagent (Takara, Japan). Quantity and quality of extracted RNA was evaluated by micro-ultraviolet spectrophotometry and visualized on 1.5% agarose gels. The first-strand cDNA was synthesized from 1 μg total RNA with random primers and reverse transcriptase M-MLV (Takara, Japan). The real-time PCR was performed by StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA) with commercial kit of SYBR Premix Ex Taq (TaKaRa, Japan). For the real-time PCR reaction, a total of 20 μL

Table 1
Results of two way ANOVA.

	Two-Way ANOVA				Two-Way ANOVA				Two-Way ANOVA		
	Interaction	Time	Density		Interaction	Time	Density		Interaction	Time	Density
crh	< 0.0001	< 0.0001	< 0.0001	Trypsin	< 0.0001	< 0.0001	< 0.0001	GPx (H)	< 0.0001	< 0.0001	< 0.0001
actha	< 0.0001	< 0.0001	< 0.0001	Lipase	< 0.0001	< 0.0001	< 0.0001	*tnf-1α* (M)	< 0.0001	< 0.0001	< 0.0001
acthβ	< 0.0001	< 0.0001	< 0.0001	Amylase	0.9247	< 0.0001	0.3304	*tnf-1α* (H)	< 0.0001	< 0.0001	< 0.0001
grα (M)	< 0.0001	< 0.0001	< 0.0001	Glucose (M)	< 0.0001	< 0.0001	0.1631	*il-1β* (M)	< 0.0001	< 0.0001	< 0.0001
grα (H)	< 0.0001	< 0.0001	< 0.0001	Glucose (H)	0.0127	< 0.0001	< 0.0001	*il-1β* (H)	< 0.0001	< 0.0001	< 0.0001
gh	0.0078	< 0.0001	< 0.0001	SOD (M)	0.0339	< 0.0001	0.0001	*il-10* (M)	< 0.0001	< 0.0001	< 0.0001
cortisol (M)	0.0105	< 0.0001	< 0.0001	SOD (H)	< 0.0001	< 0.0001	< 0.0001	*il-10* (H)	< 0.0001	< 0.0001	< 0.0001
cortisol (H)	< 0.0001	< 0.0001	< 0.0001	CAT (M)	0.0006	0.0007	0.0067	*tgf-β1*(M)	< 0.0001	< 0.0001	< 0.0001
igf1 (M)	< 0.0001	< 0.0001	< 0.0001	CAT (H)	0.0003	< 0.0001	< 0.0001	*tgf-β1*(H)	< 0.0001	< 0.0001	< 0.0001
igf1 (H)	< 0.0001	< 0.0001	< 0.0001	GPx (M)	0.0002	< 0.0001	0.0003				

P value obtained by two-way ANOVA is listed.
M: muscle; H: head kidney.

mixture was used, including 10 μL SYBR Premix Ex Taq, 0.5 μL forward and reverse primers, 0.4 μL ROX Reference Dye (50 ×), 2 μL DNA template (4-fold dilution) or no-RT control, and RNase-free water to a total volume of 20 μL.

Primers of real-time PCR were designed with reference to the known sequences of rainbow trout in NCBI and are displayed in Supplementary Table S2. PCR efficiency of each primer pair was determined by standard curves from serial dilutions (4-, 16-, 64-, 256-, and 1024-fold dilution). Two housekeeping genes (*18s* and *β-actin*) were determined in the preliminary experiments, C_T values of *β-actin* was more consistent. Thus *β-actin* was selected as the internal control of real-time PCR analysis. Analyses were based on C_T values and results were calculated relative expression to the control by the $2^{-\Delta\Delta CT}$ method [44].

2.5. Statistical analysis

Statistical analysis was performed with SPSS 16.0 Software. Two-way ANOVA was used to investigate the effects of time and density, and their interaction on the digestion and metabolism, endocrine profiles and immune parameters. Where significant differences were found, means among different density or time points were compared using Tukey's Multiple Comparison test at $P < 0.05$ significance level. Results are expressed as mean ± standard error (means ± S.E.).

3. Results

3.1. HPI axis

Density, time and their interaction significantly activated the HPI axis (Table 1). Significantly increased corticotropin-releasing hormone (*crh*) and adrenocorticotropic hormone *β* (*acthβ*) mRNA expressions were only observed within SD3 (Fig. 1A and C). Compared to day 0, expression of *acthα* mRNA showed significant increase on day 180 and 240 within trout of SD1 and SD2, and on day 180, 240 and 300 within SD3 (Fig. 1B). Among densities, trout in SD3 showed significantly increased *crh* and *acthβ* mRNA expressions from day 120 to day 300 when compared to that of SD1 (Fig. 1A and C). Expression of pituitary *acthα* of trout in SD3 was significantly higher than that of trout in SD1 and SD2 on day 240 and 300 (Fig. 1B).

Within SD1, SD2 and SD3, muscle cortisol content and glucocorticoid receptor *α* (*grα*) significantly increased to peak on day 240, and head kidney cortisol content also showed significant up-regulation from day 0–300 (Fig. 1D, E, 1F). Compared to day 0, trout within SD3 also showed significant increase of head kidney *grα* mRNA expression on day 240 and 300 (Fig. 1E). Among densities, we also observed that trout in SD3 showed significantly higher muscle cortisol content on day 240 and 300, and significantly increased muscle *grα* mRNA expression on day 180 and 240 (Fig. 1D and F). Head kidney significantly increased the cortisol content from day 120–240 in trout of SD3 with up-regulated *grα* mRNA expression on day 240 and 300 (Fig. 1E and G).

3.2. GH-IGF axis

Pituitary *gh*, muscle *igf1* and head kidney *igf1* mRNA expressions were influenced by the density, time and their interaction (Table 1). Trout within SD1 and SD2 showed significant increase of pituitary *gh* and muscle *igf1* mRNA expressions during experiment, while trout within SD3 showed low mRNA expression levels of these gene with no significant change (Fig. 2A and B). The head kidney *igf1* mRNA expression showed significant up-regulation on day 300 within SD2 and from day 180–300 within SD3 (Fig. 2C). Among densities, the *gh* mRNA expression was significantly down-regulated in trout of SD3 on day 180 and 240 when compared to that of SD1 and SD2 (Fig. 2A). Compared to trout in SD1 and SD2, trout of SD3 showed down-regulated muscle *igf1* and up-regulated head kidney *igf1* mRNA expressions on day 180, 240 and 300 (Fig. 2B and C).

3.3. Glucose content and digestive abilities

Time and interaction of density and time significantly affected the muscle glucose content, while density, time and their interaction contributed to the significant change of head kidney glucose content (Table 1). Within SD1, SD2 and SD3, we observed significantly lower muscle glucose content on day 300 when compared to the peak content on day 60 (Fig. 3A). Compared to day 0, no significant difference was observed in head kidney glucose content on day 300 within SD1, while significantly higher head kidney glucose content was observed within SD2 and SD3 on day 300 (Fig. 3B). Among densities, muscle glucose content of trout in SD3 showed significant increase on day 120 and 180, and significant decrease on day 240 when compared to those of trout in SD1 (Fig. 3A). Compared to trout in SD1, trout of SD3 showed significantly higher head kidney glucose content from day 120–300 (Fig. 3B).

Density, time and their interaction resulted in significant differences in trypsin and lipase activities (Table 1). On day 300, trout within SD2 and SD3 showed significantly decreased trypsin activity, while no significance of trypsin activity was observed within trout of SD1 when compared to that on day 0 (Fig. 4A). We also observed significantly increased lipase activity within each density from day 0–300 (Fig. 4B). Trout in SD1 showed significantly higher trypsin and lipase activities than those of trout in SD2 and SD3 on day 240 and 300 (Fig. 4A and B). Amylase activity within each density significantly increased on day 120 and 180 and then decreased to basal level on day 300 (Fig. 4C), with the significant contribution of time (Table 1). No noticeable differences in amylase activity among different densities were observed (Fig. 4C).

3.4. Antioxidant enzymes

In muscle, trout within SD1 only showed significant changes of SOD activity among day 60, 120 and 300 (Fig. 5A, 5C, 5E). Compared to day 0, trout within SD2 showed significantly higher SOD and GPx activities on day 300, and trout within SD3 showed significantly increased

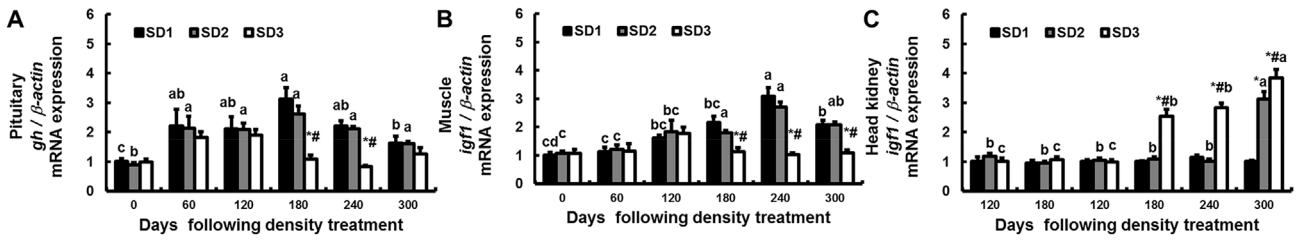

Fig. 2. GH-IGF axis of trout in different densities. Pituitary *gh* (A), muscle *igf1* (B), and head kidney *igf1* (C). Data are presented as mean ± SEM. Each point tested 4 trout (n = 4). Different letters within SD1, SD2 or SD3 indicate significant differences within each density group over all time points, * indicates significant differences between SD1 and SD2 or between SD1 and SD3 at a given time point, # indicates significant differences between SD2 and SD3 at a given time point ($P < 0.05$, two-way ANOVA, followed by Tukey's Multiple Range test). The *p*-value of density, time and interaction computed for two-way ANOVA is shown in Table 1.

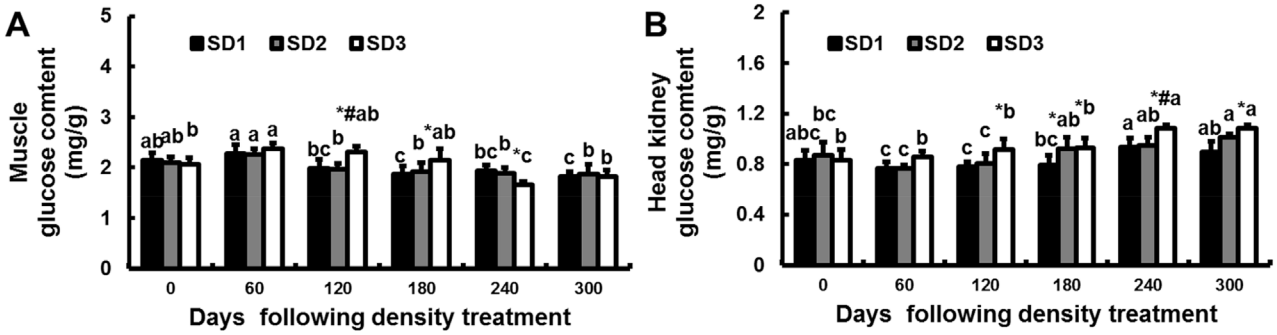

Fig. 3. Glucose content of trout in different densities. Muscle glucose (A) and head kidney glucose (B). Data are presented as mean ± SEM. Each point tested 6 trout (n = 6). Different letters within SD1, SD2 or SD3 indicate significant differences within each density group over all time points, * indicates significant differences between SD1 and SD2 or between SD1 and SD3 at a given time point, # indicates significant differences between SD2 and SD3 at a given time point ($P < 0.05$, two-way ANOVA, followed by Tukey's Multiple Range test). The *p*-value of density, time and interaction computed for two-way ANOVA is shown in Table 1.

activities of SOD on day 60, 240 and 300, CAT on day 60, and GPx on day 240 and 300 (Fig. 5A, 5C, 5E). Among densities, when compared to that of SD1, SOD, CAT and GPx activity was significantly increased in trout of SD3 on day 240, 60, as well as 240 and 300, respectively (Fig. 5A, 5C, 5E).

In head kidney, SD1 only showed significant increase of GPx activity on day 300 (Fig. 5B, 5D, 5F). Significantly increased antioxidant enzymes activities were observed within SD2 on day 240, and within SD3 on day 180, 240 and 300 (Fig. 5B, 5D, 5F). Among densities, the head kidney SOD, CAT and GPx activities were significantly increased in SD3 from day 180–300 when compared to trout of SD1 (Fig. 5B, 5D, 5F). Time, density and their interaction showed significant influences on muscle and head kidney antioxidant enzymes activities (Table 1).

3.5. Anti-inflammatory and proinflammatory cytokines

Within SD1, up-regulated *tnf-1α* and *il-1β* mRNA expressions in head kidney and muscle were observed only on day 300 (Fig. 6A–D). The up-regulations of *tnf-1α* and *il-1β* mRNAs in muscle and head kidney within SD2 and SD3 were observed after day 180, 240 or 300 (Fig. 6A, 6B, 6C, 6D). Among densities, the *tnf-1α* and *il-1β* mRNA expressions in head kidney and muscle showed no significant difference among densities on day 120, while significantly up-regulated expressions in trout of SD3 were observed on day 240 and 300 when compared to those of SD1 (Fig. 6A–D).

Trout of SD3 showed significantly up-regulated mRNA expressions of *il-10* and *tgf-β1* in head kidney and muscle from day 120 or 180, while trout within SD1 and SD2 showed up-regulations of these genes from day 180 or 240 (Fig. 6E–H). Among densities, as early as on day 120, head kidney showed significantly higher mRNA expressions of *il-10* and *tgf-β1* in SD3 when compared to those of SD1 and SD2. On day 240 and 300, the mRNA expressions of *il-10* and *tgf-β1* in muscle and head kidney of trout in SD3 were significantly higher than those of trout in SD1 (Fig. 6E–H). Density, time and their interaction contributed to the significant up-regulation of *il-10*, *tgf-β1*, *tnf-1α* and *il-1β* mRNA in SD3 on day 300 (Table 1).

3.6. The comparative analysis of neuro-endocrine-immune network during eustress and distress

Principal components analysis and heatmap were conducted to analyze the data of the neuro-endocrine-immune network on day 120 and 300. Significant separations (Fig. 7A and 7B) and colored clusters (Fig. 7C and 7D) were observed between SD3 and the other two densities, demonstrating that there were significantly different characteristics in neuro-endocrine-immune network on day 120 and 300. The Loadings plot generated from Partial Least Squares - Discriminant Analysis (PLS-DA) represents the contributions of the parameters to the differences among densities (Fig. 7E–H). Points farther away from the center or with high variable important in projection (VIP) scores contribute more than the nearby plots. On day 120, head kidney *il-10* and *tgfβ1*, *acthβ*, *crh*, and muscle *il-10* contributed to the separation of SD3 and SD1/SD2 (Fig. 7E, 7G, 7I). On day 300, the most important parameters were trypsin, lipase, amylase, head kidney cortisol and *actha* (Fig. 7F, 7H, 7J).

4. Discussion

Increase of cortisol in stressful conditions is the evolutionarily conserved mechanisms to cope with stress in animals including teleosts [27]. Magnitude and duration of the cortisol alternations are dependent on the type, intensity, duration of the stressor and the history of animals [45]. The HPI axis was persistently activated in trout of SD3, suggesting that trout were in a long-term stressful condition (Fig. 1). On day 120, despite the *crh* and *acthβ* mRNA transcriptions and the head kidney cortisol content in SD3 were significantly higher than those in SD1 and

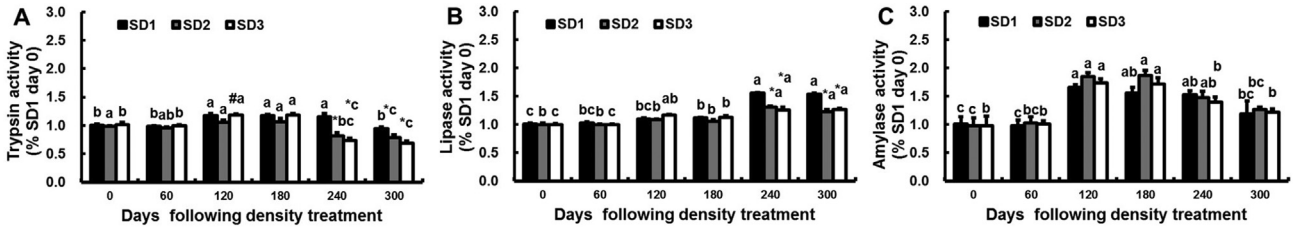

Fig. 4. Digestive ability of trout in different densities. Trypsin activity (A) and lipase activity (B) and amylase activity (C). Data are presented as mean ± SEM. Each point tested 4 trout (n = 4). Different letters within SD1, SD2 or SD3 indicate significant differences within each density group over all time points, * indicates significant differences between SD1 and SD2 or between SD1 and SD3 at a given time point, # indicates significant differences between SD2 and SD3 at a given time point ($P < 0.05$, two-way ANOVA, followed by Tukey's Multiple Range test). The *p*-value of density, time and interaction computed for two-way ANOVA is shown in Table 1.

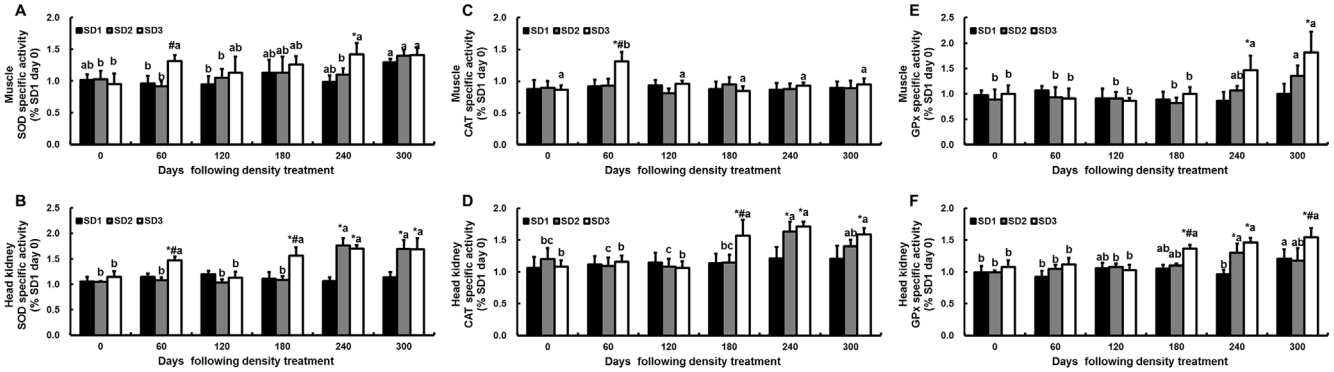

Fig. 5. Antioxidant enzyme activities of trout in different densities. Muscle SOD (A), head kidney SOD (B), muscle CAT (C), head kidney CAT (D), muscle GPx (E), head kidney GPx (F). Data are presented as mean ± SEM. Each point tested 4 trout (n = 4). Different letters within SD1, SD2 or SD3 indicate significant differences within each density group over all time points, * indicates significant differences between SD1 and SD2 or between SD1 and SD3 at a given time point, # indicates significant differences between SD2 and SD3 at a given time point ($P < 0.05$, two-way ANOVA, followed by Tukey's Multiple Range test). The *p*-value of density, time and interaction computed for two-way ANOVA is shown in Table 1.

SD2, the absolute values were low (Fig. 1). The slightly increased HPI axis was probably triggered by eustress caused by low levels of crowding. Eustress plays an important role in activation of the immune responses, which is beneficial for fish to deal with the further high levels of stress [46]. Therefore, we observed the upregulated immune parameters in trout of SD3 (discussed below, Figs. 5 and 6) and normal digestive activities for the energy supplement (Fig. 4).

During long-term exposure to mild stress, adaptation mechanisms enable fish to redistribute the energy usage and to restore the cortisol level to basal range [47]. Consistently, we observed that the HPI axis of trout in low or intermediate density (SD1 or SD2) was partially restored on day 300, consistent with previous studies of sea bass, seabream and trout with different genetic background or extremely acute crowding stress [8,34,35,48]. However, trout with increased allostatic load caused by severe and prolonged crowding stress were more likely to suffer distress [49]. Since the HPI axis plays a central role in the regulation of stressful and immune responses [50], the decreased responsiveness of the HPI axis, probably caused by chronic stress induced adaptation with cortisol desensitization [51], could disturb the endocrine feedback mechanisms, further causing the dyshomeostasis of the neuro-endocrine-immune network in trout of SD3.

Consistent with HPI profiles, we observed significantly increased muscle/head kidney glucose content in SD3. In teleost, glucose provides key energy for homeostasis restoration in the face of stressor [52,53]. We propose that when trout of SD3 suffered chronic crowding stress, glucose usage was increased due to the increased energy consumption of stress responses. The elevated glucose is also observed in other teleosts including model (zebrafish) and economic (sea bass) teleost [8,54] when they are exposed to acute crowding stress. Trout in SD3 also showed significant increase in muscle triglyceride instead of glucose from day 240–300 (Supplementary Fig. S1), indicating an increased lipid usage for energy mobilization and compensation for the increased stressful and immune responses.

The GH-IGF axis is also regulated by the HPI axis [55,56]. Previous studies have indicated that stress could increase cortisol levels, resulting in an increase in the energy used for stress coping mechanisms and a decrease in energy allotted for growth and immunity [50,57–59]. Trout in high density showed suppression of muscle GH-IGF axis (Fig. 2) and poor growth performance (unpublished data) with activated HPI axis (Fig. 1), consistent with results in sturgeon (*Acipenser sinensis*) [60]. Indeed, an intracellular cross-talk between stress, growth, and immune is observed in teleost. In the liver, cortisol triggered the transcription of specific genes including suppressors of cytokine signaling (*socs*) and phosphoenolpyruvate carboxykinase (*pepck*) [53,61]. The SOCS could further inhibit the GH signaling, suppressing the *igf1* transcription and regulating the transcriptional levels of specific cytokines, and the PEPCK induced glucose metabolism [53,61], which is closely consistent with our results in liver. However, we observed conflicting results in kidney with up-regulation of *igf1*. Several previous studies have confirmed that IGF1 is an immune modulator in fish (Yada, 2007, 2009). Based on these evidences, we propose that trout might regulate the energy redistribution via the interaction of HPI and GH-IGF

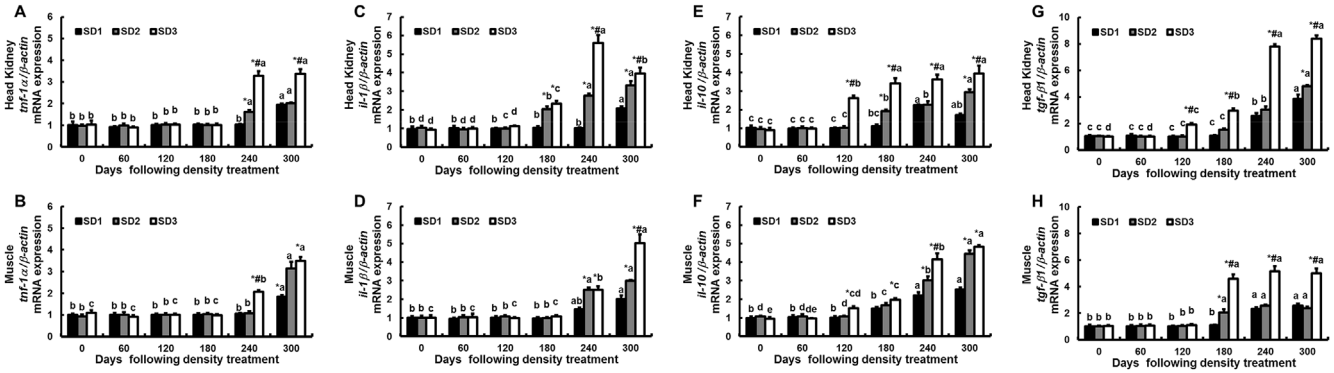

Fig. 6. Cytokine transcriptions of trout in different densities. Head kidney and muscle *tnf-1α* (A, B), head kidney and muscle *il-1β* (C, D), head kidney and muscle *il-10* (E, F), head kidney and muscle *tgf-β1* (G, H). Data are presented as mean ± SEM. Each point tested 4 trout (n = 4). Different letters within SD1, SD2 or SD3 indicate significant differences within each density group over all time points, * indicates significant differences between SD1 and SD2 or between SD1 and SD3 at a given time point, # indicates significant differences between SD2 and SD3 at a given time point ($P < 0.05$, two-way ANOVA, followed by Tukey's Multiple Range test). The *p*-value of density, time and interaction computed for two-way ANOVA is shown in Table 1.

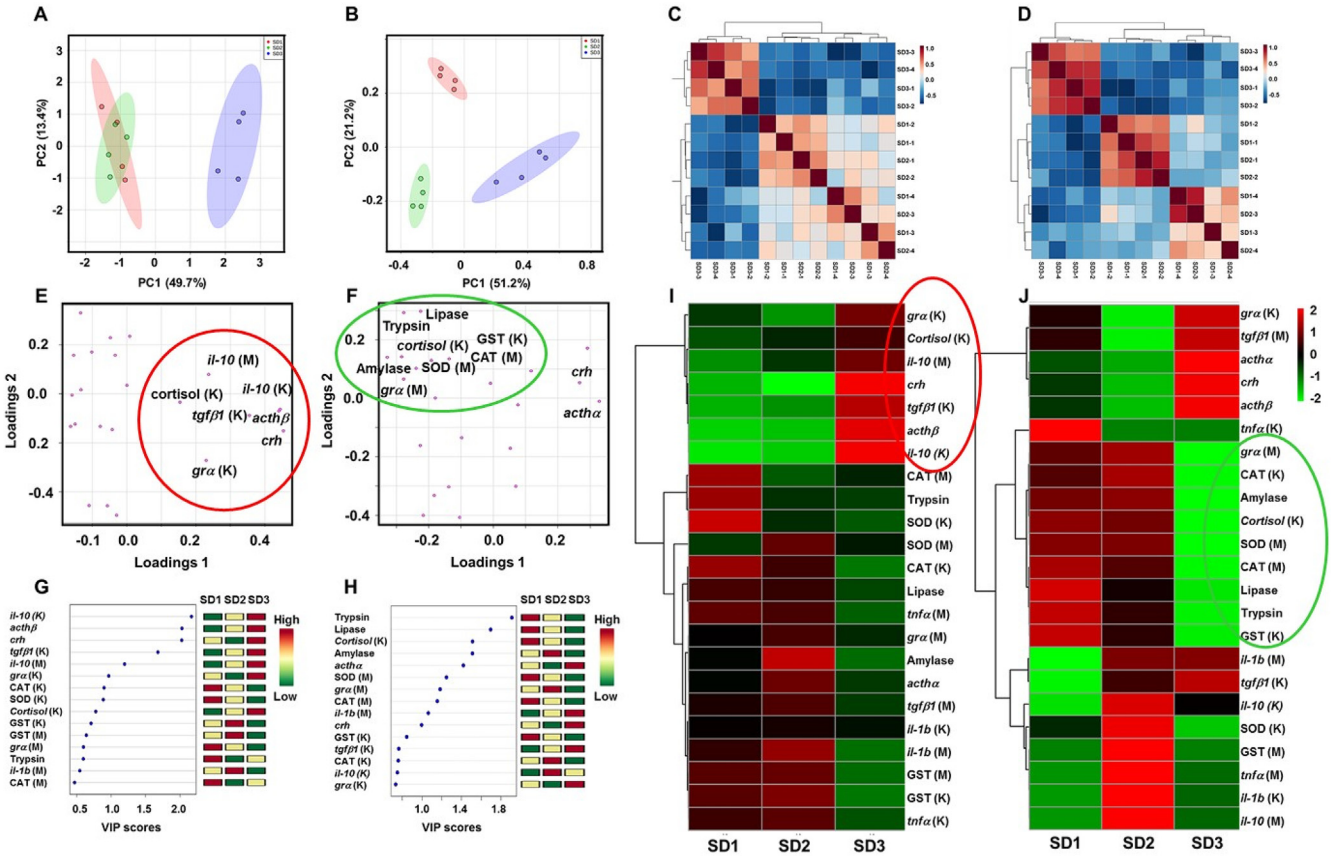

Fig. 7. Partial Least Squares - Discriminant Analysis (PLS-DA) and heatmap of neuro-endocrine-immune network. Partial Least Squares - Discriminant Analysis (PLS-DA) and heatmap of the samples from different densities on day 120 (A, C) and 300 (B, D). Loadings plot and variable important in projection (VIP) scores derived from the PLS-DA on day 120 (E, G) and 300 (F, H). Heatmap of differential parameters on day 120 (I) and 300 (J). Red color indicates up-regulation and green color indicates down-regulation. Significant separations (A, B) and colored clusters (C, D) were observed between SD3 and the other two densities, demonstrating that there were significantly different characteristics in neuro-endocrine-immune network on day 120 and 300. Points farther away from the center or with high variable important in projection (VIP) scores (VIP score $>$ 1.1 [80]) contribute more than the nearby plots (E–H). On day 120, head kidney *il-10* and *tgfβ1*, *acthβ*, *crh*, and muscle *il-10* contributed to the separation of SD3 and SD1/SD2 (E, G, I). On day 300, the most important parameters were trypsin, lipase, amylase, head kidney cortisol and *acthα* (F, H, J). Important clusters of parameters that indicated significant differences were marked as red circle (E, I) on day 120 and green circle on day 300 (F, J). M: muscle; K: head kidney. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

axis, converting the energy from growth to immune defense.

Reactive oxygen species (ROS) and the resulting oxidative stress may severely damage lipids, proteins and DNA structures and/or functions, causing cellular damage and apoptosis [62,63]. The SOD, CAT and GPx are important antioxidant enzymes, reducing the over-expression of ROS and protecting cells from oxidative damage [64]. We observed the significantly increased antioxidant enzyme levels in trout of SD3, which was consistent with previous studies [65,66], indicating that teleost could trigger an increased antioxidant defense during stress [65,67,68]. Moreover, the antioxidant responses showed organ-specific activation patterns, with an earlier increased SOD, CAT and GPx activities in head kidney (Fig. 5). We propose that low intensity of stress could initially trigger the ROS accumulation in immune organs such as head kidneys. When the intensity of stress was further increased, muscle antioxidant defensive responses were activated due to the fact that antioxidant defense of head kidney was insufficient to prevent the increased oxidative stress.

Eustress results in slight activation of the HPI axis, which has positive effects on the immune capacity by upregulating innate immune parameters, such as slightly enhanced antioxidant defenses [69–71]. Low level of oxidative stress is capable of exerting eustress by enhancing defense capacity with beneficial health effects (reviewed in Ref. [72]), enabling fishes to be more resistive to the stressful conditions when compared to those without prestress [22,46]. Consistently, we observed the increased antioxidant enzyme levels as early as on day 60 in SD3 (Fig. 5). Despite the antioxidant defense is beneficial, it results in an increased energetic consumption [73]; therefore, we observed impaired phenotypes of digestion (Fig. 4) and growth (unpublished data) on day 240 and 300. Similar results are also reported in other teleosts [65] during long-term environmental stress.

Cytokines are key regulators for the immune responses [74]. Based on previous studies in teleost [53,75–77], we selected two proinflammatory cytokines (*tnf-1α* and *il-1β*) and two anti-inflammatory cytokines (*il-10* and *tgf-β1*) to evaluate the immune responses of trout. We observed relatively low and stable expression levels of *tnf-1α* and *il-1β* with increased cortisol content on day 180 (Figs. 1 and 6), which is consistent with previous studies that cortisol inhibits proinflammatory cytokines in teleosts [78]. As the constant growth of trout, density of SD3 was around 50 kg/m^3 on day 300. Despite high expression levels of anti-inflammatory cytokines were observed, the increased intensities of crowding stress were combined with the loss of suppression resulting from decreased cortisol content, collectively contributing to the significantly up-regulated *tnf-1α* and *il-1β* expressions of trout on day 300. All these results suggested that severe and prolonged crowding stress may cause inflammation in trout.

As early as day 120, trout of SD3 showed significantly higher *il-10* and *tgf-β1* mRNA expression in head kidney. Despite a slightly increased cortisol content was observed, no oxidative stress was observed on day

120. Anti-inflammatory cytokines negatively regulate the proinflammatory cytokines induced inflammatory stress [74]. We might propose that the low levels of stress, acting as eustress, could slightly activate the HPI axis and anti-inflammatory cytokine transcriptions, enabling trout to enhanced immunity and adaptive competences with high levels of stress.

5. Conclusion

Mildly stressful situations cause eustress and higher severities cause distress [22]. During eustress and/or distress, cortisol is an important endocrine and immune regulator, regulating GH-IGF axis, glucose mobilization and cytokine transcriptions (Fig. 8) [53,61]. We observed that the eustress resulting from low levels of crowding could slightly activate the neuro-endocrine-immune network, inducing the HPI axis activation, up-regulated transcriptions of the anti-inflammatory cytokines and increased activities of antioxidant enzymes (Fig. 7E, 7G, 7I), which was beneficial for trout to deal with crowding stress. However, the long-term and high-level crowding conditions resulted in distress, causing a new set point of the homeostasis of neuro-endocrine-immune network. During distress, we observed the prolonged HPI axis activation with suppressed GH-IGF axis, up-regulated mRNA expressions of cytokines and severe ROS stress (Fig. 7F, 7H, 7J). The activation of HPI axis and immune defense could consume a large amount of energy, however, the energy supplement was decreased due to the result that digestive ability was also significantly suppressed by distress (Figs. 4 and 7). Consequently, we observed the dyshomeostasis of energy balance and neuro-endocrine-immune network of trout during long-term crowding conditions.

Despite the rearing conditions with high density in aquaculture are widely recognized as negative stressors of animal welfare and animal harvest, our studies showed the eustress resulted from low levels of crowding could slightly enhance the immune capacity of trout, which is consistent with previous studies showing that low stocking density negatively affects growth, metabolism and stressful responses of meagre (*Argyrosomus regius*) [79]. Based on these results, we might suggest an intermediate stocking density of trout culture, which will allow for full exploitation of water resource and fishery facilities with high economic

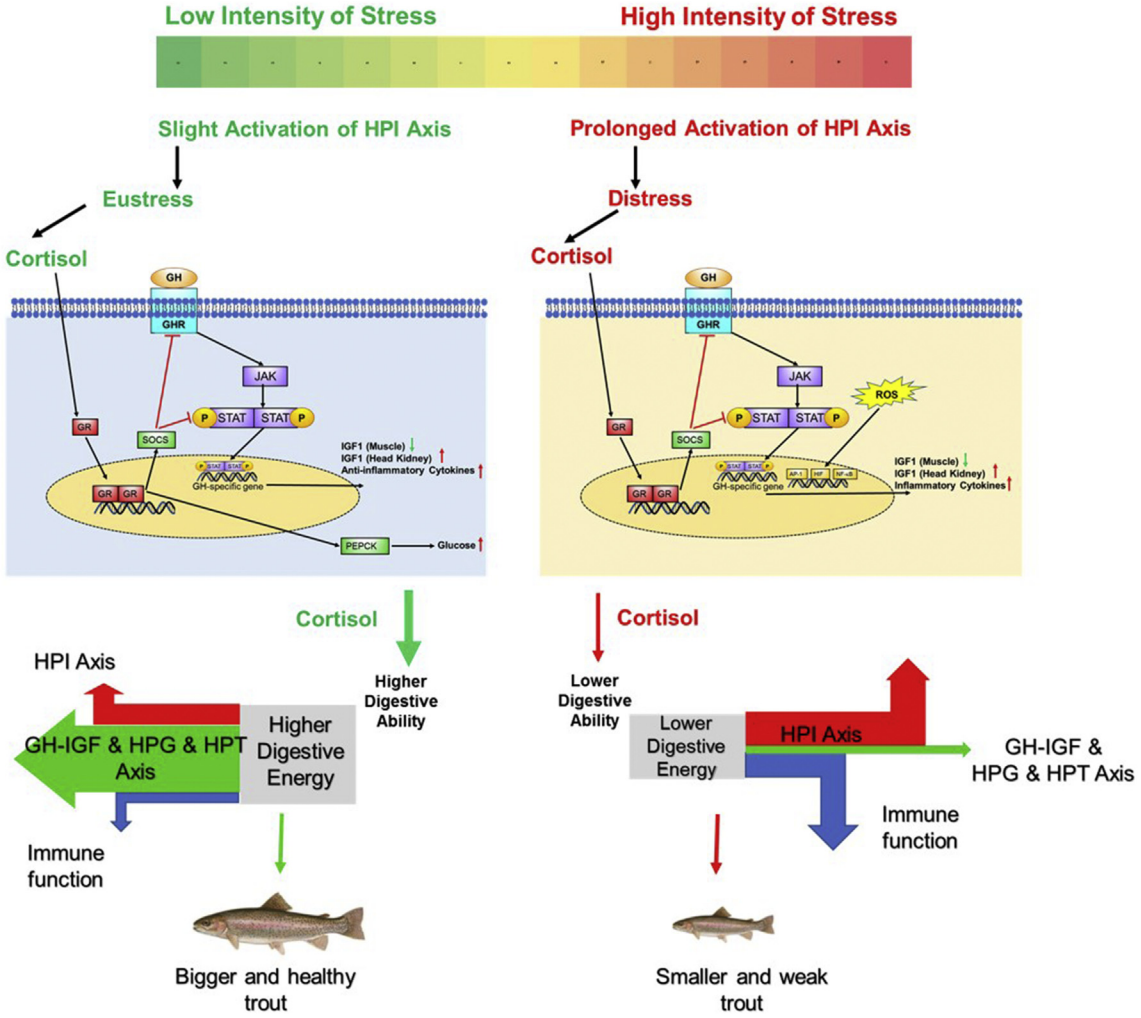

Fig. 8. A proposed model of dyshomeostasis of energy balance and reset of neuro-endocrine-immune network in trout during long-term crowding conditions. Low intensity of stress causes eustress and higher severities cause distress. During eustress and/or distress, cortisol is an important endocrine and immune regulator, regulating GH receptor binding and its intracellular signaling via SOCS and inducing glucose mobilization via PEPCK. Cortisol also regulates transcriptions of GH-specific gene including the *IGF1* and *cytokine transcriptions*. During eustress, a low cortisol level slightly inhibits GH signaling, causing glucose mobilization, increased synthesis of anti-inflammatory cytokines, and increased digestive ability. Despite the GH-IFG axis is slightly suppressed, the trout could maintain the homeostasis of neuro-endocrine-immune network by a higher energy supplement from digestive energy. However, during distress, HPI axis is prolonged activated, causing increased synthesis of proinflammatory cytokines, and severely decreased digestive ability and suppressed GH-IGF axis. The ROS stress further increases the cellular stress, causing the dyshomeostasis of energy balance and reset of neuro-endocrine-immune network. This figure is partially adjusted from previous srudies [53,61].

profits. Furthermore, trout should be divided into lower density for long-term stocking when the rearing density is approaching to 40 kg/m^3 with distress.

CRediT authorship contribution statement

Zhi-Shuai Hou: Conceptualization, Project administration, Writing - review & editing, Writing - original draft. **Hai-Shen Wen:** Conceptualization, Supervision, Writing - review & editing, Writing - original draft. **Ji-Fang Li:** Conceptualization. **Feng He:** Methodology. **Yun Li:** Methodology. **Xin Qi:** Methodology.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.10.011.

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