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# Growth under serial stocking density and transcriptome analysis of the brain in rainbow trout *Oncorhynchus mykiss*

Kouyou Fujishiro<sup>1</sup> · Sotaro Nishikawa<sup>2</sup> · Hiroshi Miyanishi<sup>2</sup>

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## Abstract

High stocking densities negatively affect fish production, even if the water quality is managed correctly. However, the mechanisms underlying the growth inhibition at high stocking densities remain unknown. This study aimed to reveal changes in gene expression in the brain that processes sensory information and to predict neuronal response changes under high stocking densities. Juvenile rainbow trout were reared at five different densities (22–180 fish/tank) for 60 days. The 180 fish group showed lower body weights than that of the 22 fish group on the 28th day, even though environmental factors such as water quality, dissolved oxygen, and feeding conditions were the same. Final fork length and condition factor results revealed growth inhibition in the high-density group. In addition, the brain transcriptomics of the 22 and 180 fish groups were analyzed. Gene set enrichment analysis based on the Kyoto Encyclopedia of Genes and Genomes was used to detect alterations in the five pathways. Steroid biosynthesis and extracellular matrix–receptor interaction pathways were downregulated in the 180 fish group, suggesting the stagnation of myelination in a high-density environment. These results offer insights into growth inhibition mechanisms and brain responses in environments where many individuals of the same fish species are present.

**Keywords** High stocking density · Growth inhibition · Rainbow trout · RNA-seq · Gene set enrichment analysis

## Introduction

In aquaculture, maintaining the number and density of fish in tanks is an important aspect of farming. Stocking fish at high densities deteriorates water quality, resulting in low feed intake and growth inhibition (Conde-Sieira et al. 2010; Li et al. 2012; McKenzie et al. 2012). Therefore, appropriate stocking density control is essential to maximize aquaculture fishery harvests. Growth inhibition owing to high-density

rearing is generally considered to be caused by poor water quality, such as accumulated nitrogen-form substances and insufficient oxygen levels, as well as stress from the high density (Piper 1970; Baker and Ayles 1990; Bagley et al. 1994; Ellis et al. 2002). However, previous studies have shown that growth inhibition occurs at high densities even though water deterioration is prevented (Papoutsoglou et al. 2005; Qi et al. 2016; Liu et al. 2017; Hayat et al. 2018; Fujishiro and Miyanishi 2023). In addition, an equal feeding rate was set for fish to consume all food, and the growth inhibition of highly stocked fish was confirmed (Mizuno et al. 2010; Salas-Leiton et al. 2010; Fujishiro and Miyanishi 2023). The causes and mechanisms for growth inhibition at high densities have been the subject of much discussion. A high stocking density is detrimental to aquaculture as it inhibits growth and is one of the factors limiting productivity. Elucidating growth inhibition at high densities can improve aquaculture productivity. Rainbow trout (*Oncorhynchus mykiss*) is a globally commercial, high-value, and popular aquaculture target fish. In fact, members of the Food and Agriculture Organization of the United Nations produced 1,987,052 t of aquacultured rainbow trout in 2022 (Global

✉ Hiroshi Miyanishi  
miyanishi@miyazaki-u.ac.jp

Kouyou Fujishiro  
gd18024@student.miyazaki-u.ac.jp

Sotaro Nishikawa  
amphysiology@miyazaki-u.ac.jp

<sup>1</sup> Department of Applied Biological Science, Interdisciplinary Graduate School of Agriculture and Engineering, University of Miyazaki, Miyazaki 889-2192, Japan

<sup>2</sup> Faculty of Agriculture, University of Miyazaki, Miyazaki 889-2192, Japan

Aquaculture Production in *Fisheries and Aquaculture*, FAO: <https://www.fao.org/fishery/en/collection/aquaculture> “Accessed 10 Mar 2025”). In addition, this species also has the second-largest inland aquaculture productivity in Japan, at 4,462 t (e-Stat: [https://www.e-stat.go.jp/stat-search/files?stat\\_infid=000040146672](https://www.e-stat.go.jp/stat-search/files?stat_infid=000040146672) “Accessed 8 Mar 2025”). As the global population continues to grow, the demand for rainbow trout is expected to remain high, highlighting the need for methods and technical advancements to enhance its production. Furthermore, with its fully sequenced genome (Berthelot et al. 2014), the rainbow trout serves as a valuable model for studying the effects of stocking density and the growth inhibition mechanisms in salmonids. However, to our knowledge, no rainbow trout studies have evaluated the effects of high density on growth with maintained water quality and a standardized amount of feed based on the tank biomass.

In this study, we focused on the effects of density solely by normalizing the feed amount and ensuring consistent water quality. Juvenile rainbow trout were reared at five different densities and fed equally relative to the biomass for 60 days to investigate the effects of growth inhibition by comparing their body weights, fork lengths, and condition factors. In addition, the transcriptome analysis was also conducted to reveal the pathway responding to high stocking densities. Our previous pseudo-high-density study revealed that visual recognition is involved in medaka density perception (Fujishiro and Miyanishi 2023). This study targeted the brain, which processes sensory information, and investigated changes in gene responses at low and high stocking densities. Although He et al. (2021) performed transcriptome analysis of the brain in highly stocked grass carp *Ctenopharyngodon idella*, no studies comprehensively analyzed gene expression in the brains of highly stocked fish whose entire genomes have been sequenced. This study leads to new insights into the growth inhibition and brain responses to social interaction under high densities.

## Materials and methods

### Fish

Rainbow trout eyed eggs of Donaldson steel-head strain were provided by the Nagano Prefectural Fisheries Experimental Station. A 12-h light and 12-h dark photoperiod cycle was maintained for fish husbandry. After hatching and yolk absorption, the fish were fed commercial feed (Otohime B2; Marubeni Nisshin Feed Co., Ltd., Japan) in one tank. Tap water was filtered using a carbon water purifier (Simple; Aquageek, Japan), which was then used for fish rearing. All fish rearing experiments and sampling were conducted in our laboratory at the University of Miyazaki. In this study,

all fish experiments were performed in accordance with the Manual for Animal Experiments prepared by the Animal Experiment Committee of University of Miyazaki.

### Growth performance analyses

Juvenile fish averaging  $1.031 \pm 0.003$  g in weight (mean  $\pm$  standard error of mean, SEM) were distributed into four experimental 18 L glass tanks. The tanks contained 22 (1.25 kg/m<sup>3</sup>), 45 (2.5 kg/m<sup>3</sup>), 90 (5 kg/m<sup>3</sup>), and 180 (10 kg/m<sup>3</sup>) fish. Black plastic partitions were installed to prevent the participants from watching the adjacent tanks. Each 18 L tank was equipped with water aeration and recirculating equipment, which was jointly cooled by the chiller (LX-180EXA, REI-SEA) to maintain the water temperature. Water overflow from each tank was gathered, partially exchanged, and then filtered by polyester sheets, gravel-packed filters, and a biofilm reactor before being directed into the chiller by the magnetic drive pump. Each tank's water temperature, dissolved oxygen (DO), and pH were monitored daily using pH and DO meters (HI 2020-01 and HI 2040-01, Hanna® Instruments). Fish were fed 2% of the total body weight of the group twice daily until the 8th day, in accordance with Nogawa and Yagisawa (2011). Some uneaten feed was observed on the evening of the 8th day, the feeding rate was then adjusted to 1.5% in the morning and 1% in the evening from the next day. No residual feed was observed subsequently. Fish were anesthetized with 250–300 ppm of 2-phenoxyethanol (Fujifilm Wako Pure Chemical). Their body weight and fork lengths were measured once a week, and the amount of feed was calibrated based on the feeding rate. The following equations were adopted to evaluate the growth parameters: condition factor (K) and specific growth rate (SGR).

$$K = BW/FL^3 \times 10^3 \quad (1)$$

$$SGR = 100 \times (\ln BW_{ave} - \ln BWI_{ave})/T \quad (2)$$

where *BW* and *FL* are the body weight (g) and fork length (cm) of the same individual, *BW<sub>ave</sub>* is the average body weight (g) in a group, *BWI<sub>ave</sub>* is the average body weight (g) of the previous measurement in the same group, and *T* is the day term from the prior measurement. On the same day of body measurement, each tank's water was collected to evaluate the NH<sub>3</sub>-N concentration using an ammonia photometer (HI-97715; Hanna® Instruments). In the 180 fish group, two fish died at the 9th and 35th days; however, the experiment was continued without replacement. After final body measurements, 22 fish from each group were randomly caught and anesthetized for dissection. The brain without pituitary glands was sampled separately and immediately frozen in liquid nitrogen.

## RNA preparation

The 22 and 180 fish groups, which showed significant differences in growth parameters were targeted to evaluate changes in brain gene expression owing to a shift in stocking density. Brain samples were homogenized, and total RNA was extracted using Isogen II (Nippon Gene Co., Ltd., Japan) following the manufacturer's protocol. The concentration and purity of the RNA were determined using a DS-11 spectrophotometer (DeNovix Inc., U.S.A.). Of the 22 randomly selected samples from each group, 17 samples with the highest body weight and A260/280 ratio > 1.9 were selected from the 22 fish group, while 18 samples with the lowest body weight and A260/280 ratio > 1.9 were selected from the 180 fish group to retain a statistical difference in body weight. The RNA was then pooled from five to six same-group samples to prepare 5 µg RNA samples. Three pooled samples per group were used for cap analysis of gene expression (CAGE)-seq.

## CAGE-seq analyses

The CAGE library preparation, sequencing, mapping, and gene expression analyses were performed by Dnaform (Kanagawa, Japan). The total RNA quality was assessed using a Bioanalyzer (Agilent Technologies Inc., U.S.A) to ensure that the RNA integrity number was > 7.0. The cDNAs were synthesized from total RNA using random primers. Ribose diols in the 5' cap structures of the RNAs were oxidized and biotinylated. The biotinylated RNA/cDNA was selected using streptavidin beads (CapTrapping). After the RNA was digested by RNase ONE/H and the adaptor ligation of both the cDNA ends, double-stranded cDNA libraries (CAGE libraries) were constructed. The CAGE libraries were then sequenced using paired-end reads of 150 nucleotides on a NextSeq 2000 instrument (Illumina). The obtained reads (CAGE tags) were mapped to rainbow trout USDA\_OmykA\_1.1 genome using STAR (version 2.7.9a).

The CAGE tag clustering was performed using the RECLU pipeline (Ohmiya et al. 2014). Tag-count data were clustered using a modified Paraclu program. Clusters with count per million < 0.1 were discarded. Regions with a 90% overlap between replicates were extracted using BEDtools (version 2.12.0). Clusters with an irreproducible discovery rate ≥ 0.1, in addition clusters longer than 200 bp were discarded.

## Pathway enrichment analysis and gene set enrichment analysis (GSEA)

A total of 93.58% of Ensembl gene IDs were converted to Entrez gene IDs using DAVID. Differentially expressed genes (DEGs) were detected using the edgeR package

(version 3.22.5). The lists of DEGs with a false discovery rate (FDR) ≤ 0.1,  $P$ -value < 0.05, and  $\log_2$  fold change (FC) < 0 or > 0 were utilized for enrichment analysis with the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) database by clusterProfiler package (Yu et al. 2012). The terms with a corrected  $P$ -value < 0.05 were considered significantly enriched. In addition, GO terms and KEGG pathways displaying significant differences between the 22 and 180 fish groups were identified using gene set enrichment analysis (GSEA). The signal-to-noise ratio was calculated as a ranking metric of GSEA (Subramanian et al. 2005).

## Gene expression analyses

To eliminate genomic contamination, the total RNA samples were treated with 1 unit of TURBO™ DNase (Thermo Fisher Scientific, U.S.A.) according to the standard protocol. 1 µg of DNase-treated total RNA was reverse transcribed using an iScript™ Reverse Transcription Supermix for RT-qPCR (Bio-Rad Laboratories, USA) and diluted 1:25. The amount of mRNA was determined by performing real-time qPCR using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, USA). Primer sets used for real-time qPCR (Online Resource, Table S1) were designed using the Real-Time PCR (TaqMan) Primer and Probes Design Tool (GenScript). On the basis of the KAPA SYBR® FAST Universal qPCR Master Mix (2X) Kit (Nippon Genetics, Japan) protocol, all real-time qPCR reactions were conducted in a 10 µL mixture consisting of 5 µL KAPA SYBR® FAST qPCR Master Mix, each with 0.02 µL of 100 µM forward and reverse primers, and 2.5 µL of diluted cDNA template. The specificity of real-time qPCR for each target gene was confirmed using the melt curve data generated by the CFX96 Touch Real-Time PCR Detection System. The fold changes of gene expression relative to the 22 fish group were calculated using the  $2^{-\Delta\Delta C_t}$  method (Livak and Schmittgen 2001). The TATA-binding protein (*tbp*) served as an internal control gene for normalization (Mahanty et al. 2017; Liu et al. 2022).

## Statistical analyses

Data are expressed as the mean ± SEM. After examining whether these data were parametric or nonparametric using the Shapiro–Wilk test (significance with  $P < 0.05$ ), the Tukey–Kramer test (significance with  $P < 0.05$ ) was used to analyze the DO, NH<sub>3</sub>-N, body weight, total length, condition factor, and Box-Cox transformed pH data as a parametric multiple comparison among groups using KyPlot 6.0 (Kyenslab). The difference in water temperature was examined using the Steel–Dwass test (significance with  $P < 0.05$ ) as a non-parametric multiple comparison.

Furthermore, to evaluate if differences in stocking density, rearing duration, and the interaction of these factors affect water quality parameters, linear regression analyses were performed using DO, NH<sub>3</sub>-N, and Box-Cox transformed pH as response variables. The explanatory variables included density (22, 45, 90, 180 fish, as continuous variable), day (0~60 day, as continuous variable), and their interaction (density × day). The analyses were conducted using the lm() function in R version 4.4.2. In addition, due to the deviation from normality in water temperature data, generalized linear models (GLMs) were applied using the glm() function in R. The explanatory variables included density, day, and their interaction. Two distributions commonly used for positive continuous data—Gamma and inverse Gaussian—were tested with a log link function to determine the most suitable model. The model selection was based on Akaike’s information criterion (AIC).

## Results

### Growth performance

The groups did not differ in terms of water temperature, DO, pH, or NH<sub>3</sub>-N (Table 1). The linear regression analyses (Table 2) showed that the rearing duration had a significant negative effect on DO and Box-Cox transformed pH ( $P < 0.001$ ). The density and the interaction (density × day) had no significant effect on DO, NH<sub>3</sub>-N, and Box-Cox transformed pH. The adjusted R<sup>2</sup> values were greater than 0.7 for all the models (DO = 0.814, NH<sub>3</sub>-N = 0.855, Box-Cox transformed pH = 0.734), indicating a good model fit. Comparative GLM analyses indicated that the inverse Gaussian distribution model yielded the lowest AIC value (416.61), indicating superior fit compared with the Gamma distribution model (414.81). The GLM results showed that rearing duration had a highly significant positive effect on water temperature ( $P < 0.001$ ). In contrast, density and the

**Table 1** The average of daily water quality parameters at different stocking densities for 60 days

	22 fish	45 fish	90 fish	180 fish	
Temperature ( °C)	12.715 ± 0.195	12.733 ± 0.197	12.702 ± 0.195	12.736 ± 0.195	$P < 0.963$
DO (mg/L)	9.186 ± 0.069	9.186 ± 0.072	9.116 ± 0.073	9.041 ± 0.078	$P < 0.500$
pH	7.140 ± 0.042	7.141 ± 0.042	7.131 ± 0.043	7.137 ± 0.044	$P < 0.998$
NH <sub>3</sub> -N (mg/L)	1.104 ± 0.315	1.143 ± 0.329	1.189 ± 0.322	1.181 ± 0.321	$P < 0.997$

Values are mean ± SEM ( $N = 9$  in each group’s NH<sub>3</sub>-N,  $N = 61$  in the other parameters). The Tukey–Kramer test was used to analyze the DO, NH<sub>3</sub>-N, and Box-Cox -transformed pH. The difference in water temperature was examined using the Steel–Dwass test

**Table 2** Summary of linear model and GLM results evaluating the effects of density, rearing duration (Day), and their interaction on water quality parameters

Response variable	Predictor	Estimate	Std. error	$P$ value	Significance
pH [Box-cox transformed]	Intercept	571,019.502	13,752.043	< 0.001	***
	Density	41.175	132.623	0.756	ns
	Day	−5860.856	395.343	< 0.001	***
	Density × day	−1.434	3.813	0.707	ns
DO (mg/L)	Intercept	10.030	0.053	< 0.001	***
	Density	0.000	0.001	0.579	ns
	Day	−0.027	0.002	< 0.001	***
	Density × day	0.000	0.000	0.117	ns
NH <sub>3</sub> -N (mg/L)	Intercept	1.756	0.451	< 0.001	***
	Density	0.000647	0.00435	0.883	ns
	Day	−0.02277	0.01352	0.102	ns
	Density × day	−7.73E−06	1.30E−04	0.953	ns
Water temperature ( °C) [GLM, inverse gaussian, log link]	Intercept	2.355	0.009165	< 0.001	***
	Density	6.55E−06	8.84E−05	0.941	ns
	Day	0.006053	0.0002765	< 0.001	***
	Density × day	1.33E−08	2.67E−06	0.996	ns

\*\*\*  $P < 0.001$ , ns = not significant ( $P \geq 0.05$ )

interaction (density x day) were not statistically significant predictors.

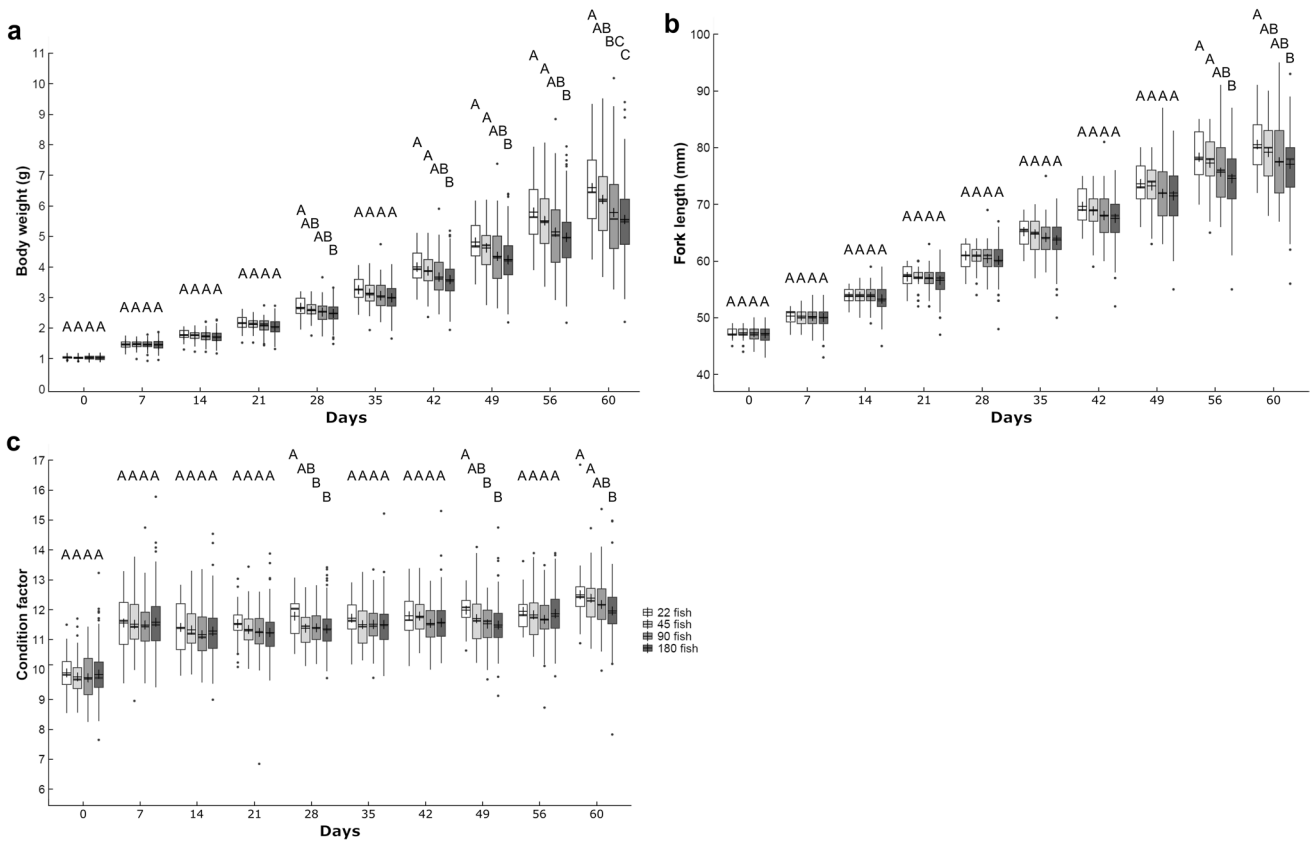
The body weight of the 180 fish group first showed a significant inhibition on the 28th day compared with that of the 22 fish group (Fig. 1a). Significant differences were also observed after this period, and the higher stocking density group had a lower mean body weight at the end of the experiment. Similarly, the fork length of the 180 fish group was retarded on the 56th and 60th days (Fig. 1b). At 28th and 49th days, the average condition factor of the 22 fish group was significantly higher than that of the 90 and 180 fish groups (Fig. 1c). On the final day, the condition factors of the 22 and 45 fish groups also varied from those of the 180 fish group. Although no statistical analysis was performed, the high-density group tended to have a decreased SGR (Table 3). Therefore, growth parameters were suppressed in the high-density group, and density-dependent growth inhibition was confirmed.

**Table 3** The weekly SGR of rainbow trout at different stocking densities for 60 days

SGR of each term	22 fish	45 fish	90 fish	180 fish
0~7 days	4.989	4.692	4.478	4.466
7~14 days	2.691	2.624	2.491	2.346
14~21 days	2.882	2.683	2.580	2.532
21~28 days	3.006	2.744	2.785	2.750
28~35 days	2.796	2.843	2.727	2.689
35~42 days	2.928	3.001	2.591	2.602
42~49 days	2.643	2.546	2.430	2.377
49~56 days	2.646	2.506	2.390	2.287
56~60 days	3.261	3.054	2.912	2.774
0~60 days	3.041	2.944	2.826	2.762

**Pathway enrichment analysis and GSEA**

Fifty-eight DEGs were subjected to GO and KEGG pathway enrichment analyses (Online Resource, Table S2).



**Fig. 1** Average body weights, fork lengths, and condition factors of rainbow trout at different stocking densities for 60 days. Each group's average value of body weight (a), fork length (b), and condition factor (c) for 60 days. The cross is average, and the horizontal line in the box is the median value. Lower and upper ends of the box represent the lower and upper quartiles (25th and 75th percentiles). The vertical

lines show the range within 1.5\*IQR (interquartile range). Different letters by the Tukey–Kramer test ( $P < 0.05$ ) indicate a significant difference between the experimental groups on the day.  $N = 22, 45, 90$  in the 22, 45, 90 fish group, respectively.  $N = 180, 179, 178$  at 0–7, 14–35, 42–60 days in the 180 fish group, respectively

The enrichment analyses did not reveal any differences between the two groups. However, the GSEA revealed the changes of the two groups in five KEGG pathways (Fig. 2a). Ribosome (omy03010) and herpes simplex virus 1 infection (omy05168) pathways were also activated at high stocking densities. Conversely, steroid biosynthesis (omy00100), extracellular matrix (ECM)-receptor interaction (omy04512), and the cytoskeleton in muscle cells (omy04820) pathways were suppressed in the 180 fish group (Fig. 2b).

## Gene expression analyses

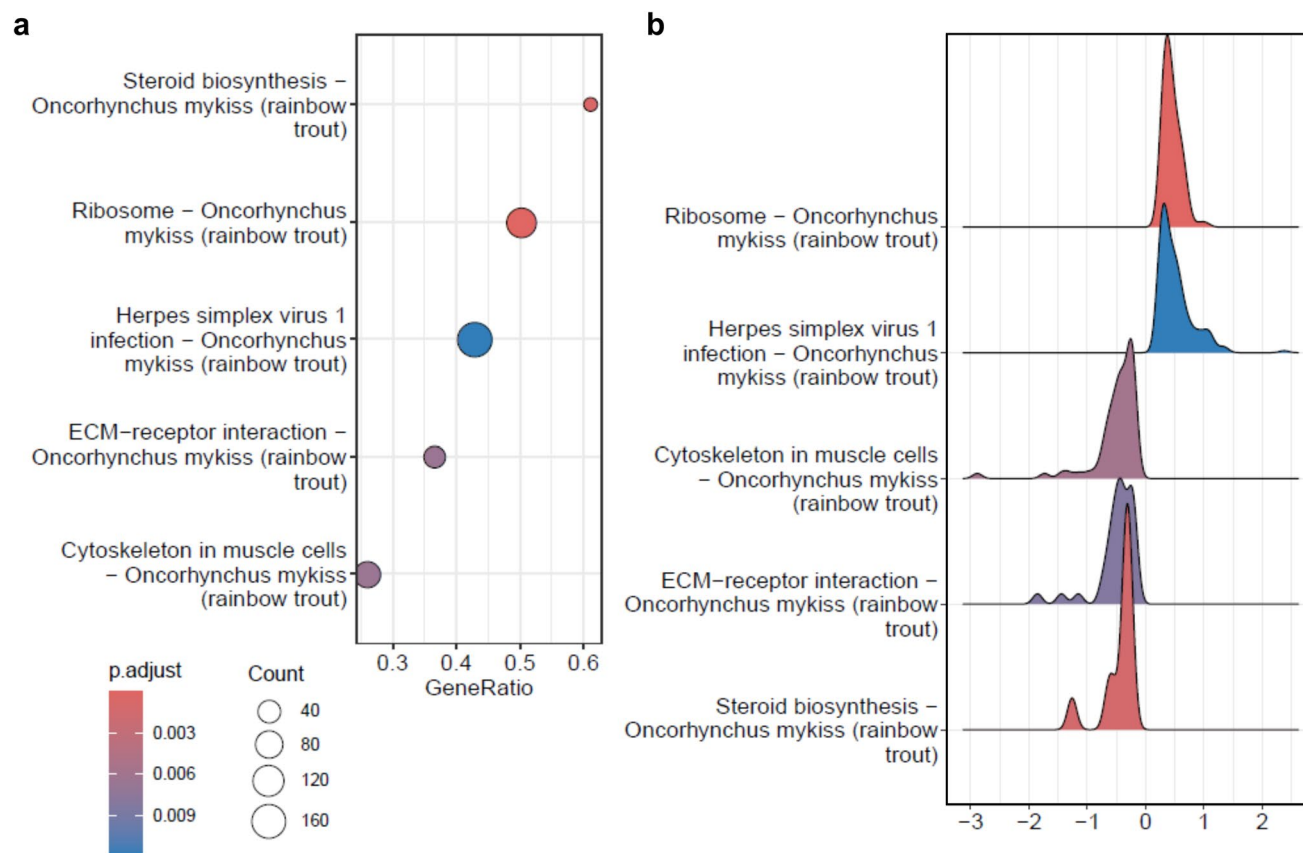
To confirm the GSEA accuracy, the expression of ranking metric top genes contributing to the significance of steroid biosynthesis and ECM-receptor interaction were assessed using real-time qPCR. Expression levels of *msm1*, *sqlea*, and *lss*, which are involved in steroid biosynthesis, were reduced under high-density conditions (Fig. 3a). Similarly, the expression of *agrin*, *dag1*, and *coll1a1b*, associated with ECM-receptor interactions, were also decreased (Fig. 3b).

No changes were observed in *tm7sf2* and *lamc1*. The results of real-time qPCR also demonstrated decreased expressions of steroid biosynthesis and ECM-receptor interaction related genes in the 180 fish group. As the synchronic decrease of both pathways was verified, high stocking density was speculated to also affected myelination. Further analysis of genes involved in myelin formation confirmed a significant downregulation of *fgfr2* and *plp1a* expression at high stocking densities (Fig. 3c).

## Discussion

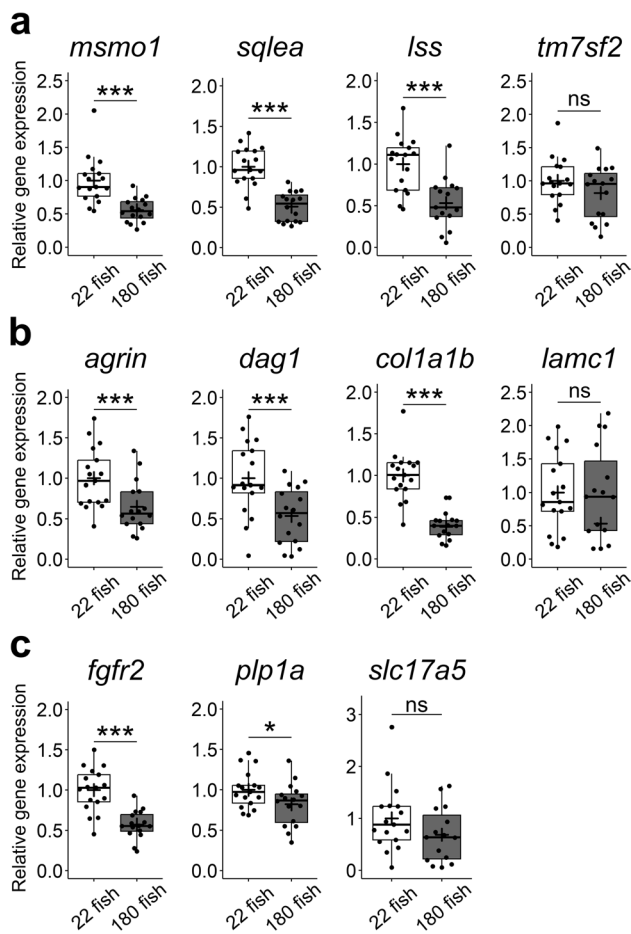
### Growth performance

Water temperature, DO, pH, and  $\text{NH}_3\text{-N}$  of each group were maintained during the stocking period with no significant differences (Table 1). The linear models also indicated that the density differences had no significant effect on water temperature, DO, pH, or  $\text{NH}_3\text{-N}$  (Table 2). The rainbow trout raised at higher stocking densities exhibited lower body



**Fig. 2** Gene set plots affected by the stocking density in the rainbow trout brain. Dot plot (**a**) and ridge plot (**b**) of enriched pathway detected by KEGG GSEA. The dot size represents the gene count. The color represents the adjusted *P*-value. The X-axis of the ridge

plot represents a log<sub>2</sub> fold change in expression for genes present in each pathway, with positive values indicating upregulated expression in the 180 fish group and negative values downregulation in the 180 fish group



**Fig. 3** Quantitative PCR confirmation of myelin-related genes and genes working in the changed KEGG pathways. The expressions of genes from steroid biosynthesis (**a**) and ECM-receptor interaction (**b**) in the KEGG pathway, and myelin-related genes (**c**). The cross is average, and the horizontal line in the box is the median value. The lower and upper ends of the box represent the lower and upper quartiles (25th and 75th percentiles). The vertical lines show the range within 1.5\*IQR (interquartile range). Following the Shapiro–Wilk test, the expressions of *tm7sf2*, *lamc1*, *plp1a*, and *slc17a5* were analyzed by the unpaired *t*-test, and the Mann–Whitney *U* test analyzed the others (\* $P < 0.05$ , \*\*\* $P < 0.001$ ).  $N = 17$  in the 22 fish group, and  $N = 16$  in the 180 fish group

weight, fork length, and condition factor, indicating growth inhibition under high stocking densities (Fig. 1). The stocking density affected fish growth; nevertheless, the amount of feed was equal to the total body mass in the tank (there was no unconsumed food, except for a small amount at day 8 in the evening). Previous studies evaluated the effects of stocking density on rainbow trout (Alanärä and Brännäs 1996; Leatherland 1993; North et al. 2006; Trenzado et al. 2006; Person-Le Ruyet et al. 2008). Previous studies assessing high stocking densities did not standardize the water quality or feed amounts per fish. This study confirmed that a higher rainbow trout stocking density resulted in significant growth inhibition, although the water quality and feed amounts

remained consistent. Corresponding with this result, growth inhibition was observed in highly stocked medaka with appropriate water quality control and the same amount of feed (Fujishiro and Miyanishi 2023). These results suggest that the energy allocation for body weight and length growth decreases at high stocking densities. Highly stocked rainbow trout consume more oxygen (Cooke et al. 2001; Larsen et al. 2012; McKenzie et al. 2012). As traits advantageous for survival and reproduction are likely heritable (Gregory 2009), the growth inhibition observed in highly stocked fish may persist as it provides advantages. For example, when the habitat expands, it may be advantageous to maintain populations with genetic diversity and to reproduce them in large numbers in the next generation. Further the investigation is required to assess the benefits of growth inhibition under crowded conditions.

### GSEA and gene expression analyses

The actions of the central nervous system (CNS) are essential for treating surrounding density information and modulating growth. Revealing the genes expressed in the rainbow trout brain at a high density can assist in understanding growth inhibition and increasing aquaculture fish production efficiency. Therefore, this study evaluated the pathways activated in the brains of highly stocked rainbow trout (Fig. 2a).

Herpes simplex virus is a pathogen, and the salmonid herpes virus 1 infects rainbow trout (Kimura et al. 1983; Wolf et al. 1978). The typical symptoms include general edema of the visceral organs, hyperemia of the liver and adipose tissue, darkened pigmentation, exophthalmia, and coelomic distension (Wolf and Smith 1981; Eaton and Hedrick 1994). Although the herpes simplex virus 1 infection pathway was upregulated in the 180 fish group, these symptoms were not observed during stocking and sampling. As the four tanks of each group shared keeping water, there is a low probability that only the 180 fish group was infected with the herpes virus. This pathway is included in the human disease category of KEGG, in addition the genes involving this pathway were selected based on mammal studies (KEGG: <https://www.kegg.jp/entry/pathway+omy05168> “Accessed 8 Mar 2025”). Accordingly, the herpes virus infection is unlikely to be responsible for activation of this pathway. Stocking density influences the immune system of rainbow trout (Yarahmadi et al. 2016; Li et al. 2024), likely reflecting changes in the immune response.

The ribosomal pathway related to mRNA translation was upregulated in the 180 fish group. Adjusting the ribosomal protein abundance is essential for adaptation to environmental changes at the translational level. Activation of the ribosomal pathway under high-density conditions suggests an increased protein synthesis rate, which is necessary for neuronal responses, cell growth, and cell division (Polymenis

and Aramayo 2015). Alternatively, considering the slower growth and ribosome activation in the 180 fish group, excessive ribosome production may cause traffic jams, collisions, and abortions, resulting in decreased protein synthesis (Subramaniam et al. 2014). The ribosome consists of ribosome proteins and rRNA (Lafontaine and Tollervy 2001). As CAGE-seq cannot detect rRNA, it is difficult to conclude that protein synthesis is activated based on the transcriptome results of this study. We hope that translating ribosomes in the brain of highly stocked fish can be quantified in the future.

The results of real-time qPCR were consistent with the findings of GSEA. The steroid biosynthesis pathway, which was suppressed in the 180 fish group (Fig. 2 and Fig. 3a), was mainly involved in the production of steroid hormone precursors, cholesterol, and vitamin intermediaries such as cholecalciferol (Glencross et al. 2015). The steroid hormone produced in the CNS is called neurosteroid. It affects anxiety, feeding behavior, and the stress response in mammals (Patchev et al. 1996; Melchior and Ritzmann 1994; Kaur and Kulkarni 2001), in addition to aggression during the non-breeding season and suppression of brain neurogenesis in fish (Soma et al. 2000; Diotel et al. 2013). It is widely presumed that aggression in salmonid groups declines as density increases, since sustaining high levels of aggression or defending a specific territory becomes inefficient or unfeasible (Cole and Noakes 1980; Bagley et al. 1994). The downregulation of this pathway, influenced by high stocking density, suggests that stimuli in a high-density environment affect fish responses and behavior through neurosteroids.

The ECM is a noncellular component that provides essential physical scaffolding for cellular elements (Frantz et al. 2010). It is composed primarily of water, proteins, and polysaccharides. During the developmental stage, the ECM participates in the proliferation and differentiation of neuronal progenitors, as well as in dendrite and axon elongation, synaptic plasticity, and neuronal connectivity (Bandtlow and Zimmermanns 2000; Long and Huttner 2019; Jovanov Milošević et al. 2014). The expressions of *agrin*, *dag1*, and *colla1b* were suppressed in high density (Fig. 3b). The suppression of *agrin* reduced filopodia density and stability on neurite, that suggests *agrin* play a role in synaptogenesis (McCroskery et al. 2006). *Agrin* is also important for axon outgrowth in motor and sensory neuron in zebrafish (Kim et al. 2007). DAG1 is an extracellular portion of the dystrophin glycoprotein complex, which intermediates the intracellular actin cytoskeleton and the extracellular environment (Ibraghimov-Beskrovnaya et al. 1992). This gene has a role in regulating dendrite complexity, axon guidance, and synaptic organizer in the CNS (Zaccaria et al. 2001; Wright et al. 2012; Pribiag et al. 2014; Bijata et al. 2015). *Colla1b* encodes the pro-alpha 1 chain, which is a major component of collagen type I, a substrate generally considered favorable

for supporting axon extension (Mneimneh and Mehanna 2021). Although this study did not histologically investigate brain neuron development, the gene suppression and deactivation of the ECM-receptor interaction pathway in the 180 fish group may be linked to the stagnation of axon elongation, synapse formation, and brain development, correlating with growth inhibition under high density.

Moreover, ECMs are necessary for normal myelin formation (Colognato et al. 2002; Barros et al. 2009; Petersen et al. 2015). Myelin comprises myelin-specific proteins, glycosphingolipids, and cholesterol, with an exceptionally high cholesterol concentration (Morell and Jurevics 1996; Morell and Quarles 1999). Approximately 70% of the dry weight of myelin is composed of lipids, with cholesterol contributing over 25% of the total lipid content (Morell and Jurevics 1996). Thus, cholesterol is required for oligodendrocytes of myelinating glial cells to express myelin genes and lap axons. As cholesterol cannot cross the blood–brain barrier (Björkhem and Meaney 2004), the steroid biosynthesis pathway in the brain is crucial for the formation of normal myelin. Synchronic inhibition of steroid biosynthesis and ECM-receptor interaction pathways implies stagnation of myelination in the rainbow trout brain under high-density conditions. Therefore, some gene expressions relating to myelination were quantified (Fig. 3c). The fibroblast growth factors receptor (FGFR) regulates the myelin-related proteins and leads to process and membrane growth for myelination (Fortin et al. 2005; Furusho et al. 2017, 2012; Rajendran et al. 2018). The *fgfr2* is expressed at mature oligodendrocytes, and the deletion of the expression at oligodendrocytes made myelin sheaths thinner (Ishii et al. 2014). The brain of a highly stocked rainbow trout had higher *fgfr2* expression, which mediates myelination (Fig. 3c). *Plp1* encodes the major CNS myelin isoproteins, PLP1, and DM20 (Weimbs and Stoffel 1994). These transmembrane proteins comprise approximately 50% of the total protein content in CNS myelin (Eng et al. 1968; Lees and Brostoff 1984). In the *plp1*-deficient mice, the apposition of the extracytoplasmic surfaces and the intraperiod dense line was absent. This disrupted assembly of the myelin sheath results in a marked reduction in the conduction velocity of CNS axons, leading to impaired neuromotor coordination and behavioral abnormalities (Boison and Stoffel 1994). The mutant zebrafish, which lack an enzyme for isoprenoid and cholesterol synthesis, results in a lack of myelin gene expression including *plp1a* and axon wrapping (Mathews et al. 2014). Consistent with findings from a previous study on fish, *plp1a* expression was suppressed in the 180 fish group (Fig. 3c), together with the downregulation of genes involved in the steroid biosynthesis pathway. Furthermore, *dag1*, which decreased in the 180 fish group, is expressed by the oligodendrocytes, and its knockdown in

cultured oligodendrocyte precursor cells leads to impaired differentiation and decreased expression of myelin-specific proteins (Colognato et al. 2007). These results raise the possibility that stable maintenance of myelin in highly stocked fish is compromised due to suppressed steroid biosynthesis in the brain. Myelin accelerates the propagation of saltatory potential by wrapping axons for insulation (Tasaki 1939; Nave and Werner 2014). For example, hypomyelination in zebrafish delays the startle response to acoustic stimuli, indicating the role of myelin in the neural circuit function control (Madden et al. 2021). Additionally, myelination is regulated by neuronal activity in fish and mammals (Gibson et al. 2014; McKenzie et al. 2014; Hines et al. 2015), and its role is proposed to optimize the timing of spikes in specific circuits (Ford et al. 2015; Arancibia-Cárcamo et al. 2017). The decrease in brain steroid synthesis, along with the suppression of ECM-related pathways affecting synapse formation, actin elongation, and myelin maintenance under high-density conditions, may alternate the velocity of electrical impulses and/or neurotransmission. In addition to delayed brain development resulting from growth stagnation, these effects may contribute to previously observed behavioral alterations under high-density conditions (Cole and Noakes 1980; Bagley et al. 1994; Larsen et al. 2012; McKenzie et al. 2012). A histological analysis of brain myelination and behavioral evaluation in highly stocked rainbow trout is required to provide valuable insights into the effects of fish stocking densities.

This study demonstrated that the growth of highly stocked rainbow trout stagnated despite the absence of water quality deterioration, even when the same amount of feed was provided in proportion to biomass. The GSEA identified the effects of stocking density on the rainbow trout brain in five KEGG pathways. Results from GSEA and real-time qPCR indicated synchronized inhibition of the steroid biosynthesis and ECM–receptor interaction pathways, suggesting hypomyelination in the brain. In addition, myelin-related genes were downregulated in the 180 fish group. These findings imply that, in response to high stocking density, the fish brain modulates neural circuit transmission accordingly. Overall, this study demonstrated density-dependent growth inhibition in juvenile rainbow trout. To our knowledge, this is the first report to describe transcriptomic pathway alterations in rainbow trout brains reared at high stocking densities. These findings provide a foundation for future research aimed at elucidating the mechanisms underlying growth inhibition under high-density conditions.

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