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Phenethylamine Is a Potential Density Stress Pheromone in Turbot (*Scophthalmus maximus*)

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Abstract: Pheromones play a vital role in regulating fish behavior, including reproduction, aggregation, hazard recognition and food location. To gain a better understanding of chemical communication in fish produced by density changes, this study analyzed the metabolites released by turbot (*Scophthalmus maximus*) under different stocking densities. The experiment was conducted at low (LD: 3.01 kg/m^2), medium (MD: 6.62 kg/m^2) and high (HD: 10.84 kg/m^2) densities for 15 days. High-throughput non-targeted metabolomics (LC-MS/MS) was used to identify variations in metabolites released into the aquatic environment by turbot at different densities. Results showed that 29 and 47 metabolites were significantly upregulated in the MD and HD groups, respectively, compared with the LD group. Among them, hexadecanedioic acid, xanthine, phenethylamine, proline and styrene were significantly upregulated in the MD vs. LD, HD vs. MD and HD vs. LD. The VIP diagram of OPLS-DA alignment showed that phenethylamine was the most important metabolite shared by MD vs. LD, HD vs. MD and HD vs. LD. Key gene changes in the GH/IGF-1 signaling pathway, HPI axis of turbot were studied using qRT-PCR for density treatment. The results demonstrated that the expression of GH, GHR and IGF-1 was significantly lower, while the expression of CRH and ACTH was higher in the HD group. Additionally, plasma levels of cortisol, glucose, triglycerides and T_3 were also highest in the HD group compared with the LD and MD groups. Phenylethylamine concentration was positively correlated with the HPI axis and negatively correlated with the GH/IGF-1 signaling pathway. To investigate the impact of phenethylamine accumulation on turbot, an acute treatment experiment with phenethylamine was set up. Its concentration in the aquatic environment was set at 0 (CON), 10^{-7} (LP) and 10^{-5} (HP) mol/L via exogenous addition, and turbot were exposed to these environments for 2 days. There was a high degree of concordance between the GH/IGF-1 signaling pathway (GH, GHR, IGF-1), HPI axis (CRH, ACTH) and plasma physiological changes (cortisol, glucose, triglycerides, T_3) in the phenethylamine-treated group and the density-treated group. Therefore, accumulation of phenethylamine with increasing stocking density may be a potential cause of density stress. Phenylethylamine has a dose-dependent and trace effect as a pheromone.

Keywords: pheromone; phenethylamine; metabolomics; *Scophthalmus maximus*; stocking density

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Key Contribution: Accumulation of phenethylamine with increasing stocking density may be a potential cause of density stress. Phenylethylamine has a dose-dependent and trace effect as a pheromone.

1. Introduction

Recently, due to the gradual depletion of fishery resources, industrial recirculating aquaculture has been developing rapidly. Industrial recirculating aquaculture raises the stocking density by controlling feed, water quality, the environment and other factors, so as to achieve the purpose of improving aquaculture benefits [1]. However, high stocking density is widely recognized as a stressor that affects the welfare of aquatic animals; it can cause physiological disorders, immunosuppression and growth inhibition in fish [2,3]. Numerous studies have demonstrated that high stocking density has a negative impact on the survival rate, antioxidant capacity and feed utilization efficiency of turbot (*Scophthalmus maximus*) [4–7]. Density stress is commonly perceived as a consequence attributed to insufficient resources (e.g., low oxygen) or an excess accumulation of metabolites (e.g., high ammonia) [8,9]. However, chemical communication under high stocking density has been largely neglected.

In natural environments, many organisms use chemical signals to learn information about their surroundings, especially aquatic organisms, which live in low-light water environments where chemical signals are sometimes more critical than visual and auditory signals [10]. Fujimoto et al. showed bile from reproductively mature male largemouth bass (*Micropterus salmoides*) attracts conspecific females [11]. Kamio et al. isolated N-acetylglucosamino-1,5-lactone, a substance that causes courtship behavior in males, from the urine of mature molting female blue crabs (*Callinectes sapidus*) [12]. Mathuru et al. discovered that chondroitin sulfate can be perceived by zebrafish (*Danio rerio*) olfaction and elicit significant startle responses [13]. Pfuderer et al. indicated crowding factors are substances released by fish under crowded conditions that inhibit their growth and reproduction [14]. Roales's study speculated that growth inhibitory factors released from crowded fish may affect the thyroid gland, leading to fat mobilization in the tissues and, thus, lowering the total fat in these animals [15]. Mounting evidence indicates that chemical signals exert influences on the reproduction, growth and even survival of aquatic animals [16].

Pheromone-related substances (such as stress hormones and chemical alarm signals) may accumulate with increasing fish stocking density [17,18]. For example, Ruane and Komen (2003) found that cortisol concentrations in water increased when the loading density of carp (*Cyprinus carpio*) increased [18]. Although most pheromones in fish remain unidentified, the few that are structurally determined are mainly low molecular metabolites such as bile salts, F-series prostaglandins, amino acids and gonadal steroids, which can be detected using metabolomics [19]. Metabolomics is a systems approach to studying the small, endogenous metabolites in organisms [20]. It can detect changes in the metabolome brought on by external or internal stressors [21]. Because of its ability to perform high-throughput chemical analysis without the necessary purification steps, metabolomics (in addition to targeted screening and bioactivity-guided fractionation) has emerged as a novel approach to identify pheromones [22–24].

In this study, by setting different stocking densities of turbot, metabolomics was used to detect the accumulation of turbot metabolites at different densities, study the correlation between metabolite accumulation and the physiological level of turbot, screen potential pheromone substances and preliminarily analyze and verify whether pheromones could be a potential source of density stress, so as to provide a new idea for the mechanism of the generation of density stress in industrialized aquaculture of marine fish.

2. Materials and Methods

2.1. Experimental System and Experimental Design

The experiment was conducted at the Weihai Institute of Marine Biological Industry Technology, China, and fish treatment was approved by the Animal Protection and Utilization Committee of the Institute of Oceanography, Chinese Academy of Sciences.

Turbot was obtained from Guoxin Oriental recirculating water culture base and reared in recirculating aquaculture systems (RASs) for 15 days to acclimatize to the experimental environment. The experimental area was equipped with three recirculating aquaculture systems, each comprising three replicated tanks (1 m^3), three whirl-separators, a mechanical microfilter, a protein separator, a decarbonization tower, a moving-bed biological filter and UV disinfection. Healthy, active and non-traumatized turbot were selected for the experiment.

Pre-experimentation, with reference to the stocking densities used by Liu et al., it was found that there was a significant difference between the experimental system and the actual production of stocking density stress [6]. Serious stress already existed in the system as high as 14 kg/m^2, leading to death and food stoppage in the high stocking density group, so we reduced the density for the experiment according to the actual situation. Density experiments: a total of 510 fish (average individual weight 136.12 ± 27.71 g) were reared for 15 days under three stocking densities: low density (LD) with 25 fish per tank (3.01 kg/m^2 at initial density), medium density (MD) with 55 fish per tank (6.62 kg/m^2 at initial density) and high density (HD) with 90 fish per tank (10.84 kg/m^2 at initial density). Each density was tested in triplicate.

Phenethylamine acute treatment experiments: fish (6 per tank) were exposed to different concentrations of phenethylamine (mol/L): 0 (control, CON), 10^{-7} (low phenethylamine, LP), 10^{-5} (high phenethylamine, HP) for 2 days. Each concentration was tested in triplicate.

Fish were fed a commercial pellet diet (53% crude protein, 12% crude lipids, 16.0% crude ash, 4.0% crude fiber, 12% water, 0.5% P, 2.3% lysine) at 0.5% feeding rate twice daily. Daily recordings of water parameters were taken at 09:00 am., including temperature, dissolved oxygen (DO), salinity and pH, using a handheld multi-parameter water quality analyzer (YSI Incorporated, Yellow Springs, OH, USA), and the content of total ammonia (TAN) and nitrite (NO_2^-) were measured using Nessler's reagent colorimetric method and the N-1-Naphthylethylenediamine photometric method (GB 13580.7–92), respectively [25,26]. During the experiment period, other water quality parameters were maintained at appropriate levels for turbot. Specifically, dissolved oxygen, pH, temperature, salinity, TAN and NO_2^- concentration varied between 7.01 and 7.12 mg/L, 7.41 and 7.46, 15.8 and 16.5 °C, 29.63 and 31.56 %, 0.23 and 0.29 mg/L and 0.07 and 0.13 mg/L, respectively. The photoperiod was maintained at 12 h light/12 h dark.

2.2. Sample Preparation

To compare the composition of metabolites released into the aquatic environment by turbot at different densities (aquatic environment metabolome), we used a rational sample collection method. Water was collected from each replicate tank until 100 L, rapidly filtered through a filter pump onto glass fiber filter paper and then the membranes were stored at −80 °C until extraction. After the experiment, all fish were fasted for 24 h. And then, three fish were randomly collected from each culture tank (nine fish per group) and anesthetized with tricaine methane sulfonate (MS-222, Sigma Diagnostics INS, St. Louis, MO, USA) at 40–45 mg/L. Blood was obtained from the tail vein using a syringe and collected in sodium heparin anticoagulation tubes. The collected blood samples were centrifuged at 3000 rpm for 10 min to obtain plasma, which was then stored at −80 °C. Immediately after blood collection, the liver, hypothalamus and pituitary gland were removed from each fish, immediately frozen in liquid nitrogen and stored at −80 °C for gene expression analysis.

2.3. Determination of Biochemical Parameters

Plasma glucose, triglyceride, cortisol and triiodothyronine (T_3) levels in fish were measured using commercial kits (#F006-1-1, #A110-2-1, #H094-1-1 and #H222-1-1) according to the instructions for use. All commercialized kits were purchased from Nanjing Jiancheng Institute of Biological Engineering (Nanjing, China).

2.4. RNA Extraction and qPCR

Total RNA was isolated from liver, hypothalamus and pituitary gland samples using a TRIzol reagent (Trans-Gen Biotech, Beijing, China). RNA concentration and purity were measured using a Nanodrop 2000 spectrophotometer (Gene Company Limited, Hong Kong, China), and purity was calculated using the 260/280 nm optical density ratio (purity: 2.0 ± 0.1). Reverse transcription of RNA into cDNA was then performed using the Evo M-MLV Mix Kit (Hunan Accurate Biomedical Technology Co., Changsha, China). Primers were designed using Primer Premier 5.0 and NCBI online website. The primer sequences used are listed in Table 1. The qPCR was conducted using an SYBR Green Premix Pro Taq HS qPCR Kit (Hunan Accurate Biomedical Technology Co., Changsha, China) with a 20 μL reaction solution on a CFX Connet Real-Time PCR System (Bio-Rad, Beijing, China). CRH, GH, IGF-1 and GHR thermal cycling conditions were 95 °C for 15 min, followed by 35 cycles of 95 °C for 15 s and 58 °C for 60 s. ACTH thermal cycling conditions were 95 °C for 15 min, followed by 35 cycles of 95 °C for 15 s and 60 °C for 60 s. Amplification specificity was validated via melting curve analysis. Melting curve analysis was also performed to examine whether each primer set amplified a single product. Three samples were assayed for each group, and all reactions were conducted in triplicates. Relative expression levels were calculated using the Pfaffl method [27].

Table 1. List of primers used for quantitative real-time PCR analysis.

Gene Name	Primer Sequence (5′–3′)	Annealing Temperature (°C)	Amplic on Size (bp)
IGF-1	F: TCGTGGACGAGTGCTGCTT R: CCGCCTTGCTAGTCTTGG	58	81
ACTH	F: TGTGGCTATTAGTGGCTGTGG R: CCTGGCAGTTCGGATTCTC	60	81
GH	F: AATAACCACGAGACACAACGCA R: GAGAACTCCCAAGACTCAACCAA	58	80
CRH	F: CCTCCTCTAACGATTGAAGATTCC R: AGGGCTGTCAATAGCTCGAC	58	123
GHR	F: ACACGTCCATTTGGATCCCC R: GCTCCCAGTTGACCATGACA	58	183
β-actin	F: TGAACCCCAAAGCCAACAGG R: GAGGCATACAGGGACAGCAC		107

2.5. Metabolite Extraction and UHPLC-MS/MS Analysis

For metabolite extraction, take the filter membrane sample in an EP tube, add 1000 μL of 80% methanol aqueous solution and put it into liquid nitrogen for 5 min; thaw on ice, vortex for 30 s, sonicate for 6 min, centrifuge for 1 min at 5000 rpm and 4 °C, take the supernatant into a new centrifuge tube, lyophilize into dry powder, add 60 μL of 10% methanol solution to dissolve and feed into LC-MS for analysis.

UHPLC-MS/MS analyses were performed using a Vanquish UHPLC system (ThermoFisher, Dreieich, Germany) coupled with an Orbitrap Q Exactive™ HF-X mass spectrometer (Thermo Fisher, Dreieich, Germany) in Gene Denovo Co., Ltd. (Guangzhou, China). Samples were injected onto a Hypesil Gold column (100 × 2.1 mm, 1.9 μm) using a 17 min linear gradient at a flow rate of 0.2 mL/min. The eluents for the positive polarity mode were eluent A (0.1% FA in Water) and eluent B (Methanol). The eluents for the negative polarity mode were eluent A (5 mM ammonium acetate, pH 9.0) and eluent B (Methanol). The solvent gradient was set as follows: 2% B, 1.5 min; 2–100% B, 12.0 min;

100% B, 14.0 min; 100–2% B, 14.1 min; and 2% B, 17 min. Q Exactive^{TM} HF-X mass spectrometer was operated in positive/negative polarity mode with spray voltage of 3.2 kV, capillary temperature of 320 °C, sheath gas flow rate of 40 arb and aux gas flow rate of 10 arb.

2.6. Data Processing and Metabolite Identification

The raw data files generated by UHPLC-MS/MS were processed using Compound Discoverer 3.1 (CD3.1, Thermo Fisher, Dreieich, Germany) to perform peak alignment, peak picking and quantitation for each metabolite. The main parameters were set as follows: retention time tolerance, 0.2 min; actual mass tolerance, 5 ppm; signal intensity tolerance, 30%; signal/noise ratio, 3; and minimum intensity, 100,000. After that, peak intensities were normalized to the total spectral intensity. The normalized data were used to predict the molecular formula based on additive ions, molecular ion peaks and fragment ions. And then peaks were matched with the mzCloud (https://www.mzcloud.org/ (accessed on 1 March 2023)), mz Vaultand Mass Listdatabase to obtain the accurate qualitative and relative quantitative results. Statistical analyses were performed using the statistical software R (R version R-3.4.3), Python (Python 2.7.6 version) and CentOS (CentOS release 6.6). When data were not normally distributed, normal transformations were attempted using the area normalization method.

2.7. Statistical Analysis and Pathway Analysis

The collected metabolites were annotated using the Human Metabolome database (http://www.hmdb.ca/ (accessed on 1 March 2023)) and the KEGG database (http://www.genome.jp/kegg/ (accessed on 1 March 2023)). The R package gmodels was used to perform principal component analysis (PCA) on the data, and the R language ropls package was used to perform supervised orthogonal partial least squares-discriminant analysis (OPLS-DA). The OPLS-DA model was further validated with cross-validation and permutation test. For cross-validation, the data were partitioned into seven subsets, where each of the subsets was then used as a validation set. A variable importance in projection (VIP) score of (O)PLS model was applied to rank the metabolites that best distinguished between two groups. The threshold of VIP was set to 1. In addition, T-test was also used as a univariate analysis for screening differential metabolites. Those with a p value of t-test < 0.05 and VIP ≥ 1 were considered differential metabolites between two groups.

Statistical analyses comprised one-way ANOVA, followed by Tukey's test, using IBM SPSS software (version 20.0) to examine significant differences between the groups. A significance level of $p < 0.05$ was used in all analyses. All data are shown as the means $\pm$ standard error (S.E.) of the treatments.

3. Result

3.1. Metabolomics Principal Component Analysis (PCA) at Different Densities

As a non-supervised multivariate data analysis method, PCA is always used to give a comprehensive view of the clustering trend for multidimensional data [28]. To screen for characteristic metabolites with significant concentration changes, the PCA approach was utilized to conduct a model with the ES+ and ES− data, respectively. Unsupervised PCA showed LD and MD were significantly differentiated, and their contribution rates were 33.7% and 27.3% (POS) and 31.7% and 26% (NEG), respectively (Figure 1); LD and HD were significantly differentiated, and their contribution rates were 39.2% and 30.7% (POS) and 58.1% and 10.9% (NEG), respectively (Figure 1); and MD and HD were significantly differentiated, and their contribution rates were 35.8% and 27% (POS) and 47.1% and 18.9% (NEG), respectively (Figure 1). It indicated that significant changes in aquatic environment metabolomes occurred at different densities.

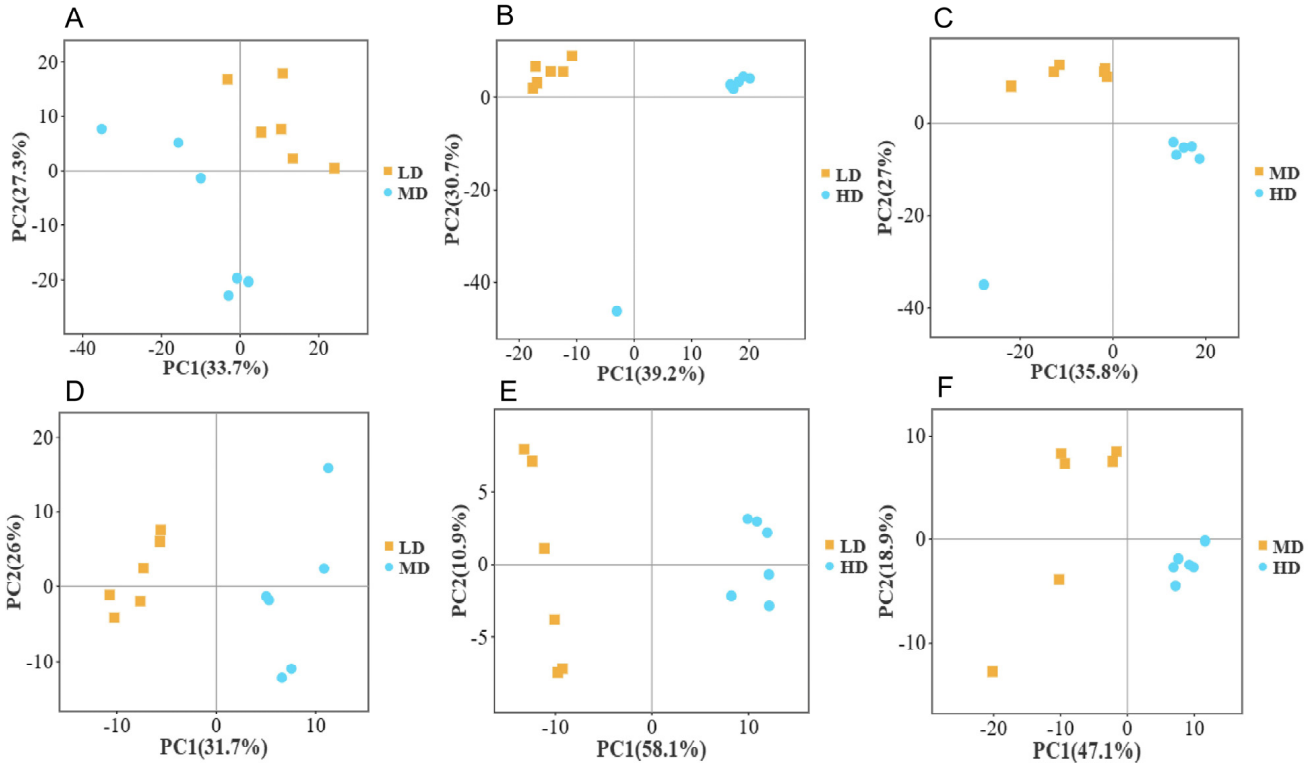

Figure 1. PCA for the metabolite profiles of samples from aquatic environments at different stocking densities. (**A**) LD vs. MD in positive ion mode; (**B**) LD vs. HD in positive ion mode; (**C**) MD vs. HD in positive ion mode; (**D**) LD vs. MD in negative ion mode; (**E**) LD vs. HD in negative ion mode; (**F**) MD vs. HD in negative ion mode.

To maximize the discrimination between different density treatments, we employed OPLS-DA to identify differences in metabolites. In the positive ion mode, the OPLS-DA score plots of LD vs. MD, LD vs. HD and MD vs. HD had cumulative values of R2X at 76.2%, 75.8% and 55.2%; R2Y at 89.3%, 95.5% and 98.3%; and Q2 at 76.8%, 63.5% and 70.5%, respectively (Table 2). In negative ion mode, the OPLS-DA score plots of LD vs. MD, LD vs. HD and MD vs. HD had cumulative values of R2X at 70.9%, 94.0% and 87.2%; R2Y at 92.4%, 98.4% and 98.3%; and Q2 at 76.9%, 96.7% and 90.2%, respectively (Table 2). R2X and R2Y denote the explanation rate of the proposed model for the X and Y matrices, respectively, and Q2 denotes the predictive ability of the model. The closer the three indicators are to one, the more stable and reliable the model is. Q2 > 0.5 indicates that the model has good predictive ability. The results indicate that the data and instrumental analysis system of this study are reliable and stable.

Table 2. OPLS-DA model validation parameters. R2X and R2Y denote the explanation rate of the proposed model for X and Y matrices, respectively, and Q2 denotes the predictive ability of the model.

Comparison Group Name	R2X	R2Y	Q2
LD vs. MD.POS	0.762	0.893	0.768
LD vs. MD.NEG	0.709	0.924	0.769
LD vs. HD.POS	0.758	0.955	0.635
LD vs. HD.NEG	0.94	0.984	0.967
MD vs. HD.POS	0.552	0.983	0.705
MD vs. HD.NEG	0.872	0.983	0.902

3.2. Differential Metabolites at Different Densities

In the positive ion mode, there were 26 significant differential metabolites (SDMs) (upregulated (up): 15, downregulated (down): 11) in the LD vs. MD group, 51 SDMs (up: 33, down: 18) in the LD vs. HD group and 50 SDMs (up: 34, down: 16) in the MD vs. HD group (Figure 2A). Eight SDMs could be identified in the LD vs. MD, LD vs. HD and MD vs. HD groups; among them, the contents of phenethylamine, proline and styrene increased with the increase in stocking density (Figure 2A). In the negative ion mode, there were 27 SDMs (up: 14, down: 13) in the LD vs. MD group, 17 SDMs (up: 14, down: 3) in the LD vs. HD group and 11 SDMs (up: 9, down: 2) in the MD vs. HD group (Figure 2B). Two SDMs could be identified in the LD vs. MD, LD vs. HD and MD vs. HD groups. Among them, the contents of xanthine and hexadecanedioic acid increased with the increase in stocking density (Figure 2B). Variable importance in projection (VIP) scores ranked by partial least square discriminant analysis (PLS-DA) are shown in Figure 3. The top three most important metabolites were gamma-glutamylleucine, phenethylamine and N-benzylformamide in the LD vs. MD group. The top three most important metabolites were oleamide, arachidonoyl amide and phenethylamine in the LD vs. HD group. The top three most important metabolites were Phenethylamine, guanine and 2-amino-1,3,4-octadecanetriol in the MD vs. HD group.

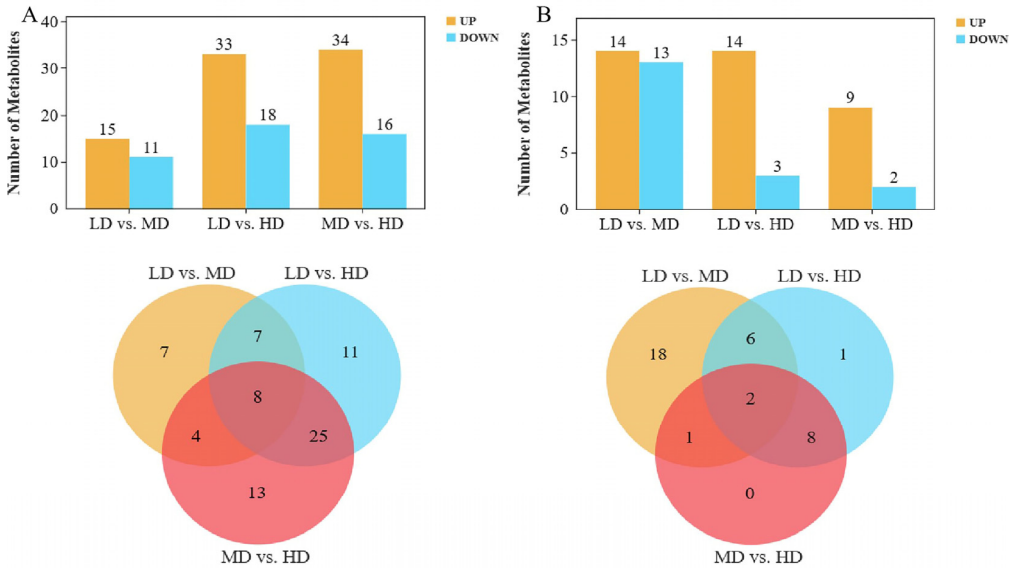

Figure 2. Identification of significantly differentially abundant metabolites in LD vs. MD, LD vs. HD and MD vs. HD for positive ion mode (**A**) and negative ion mode (**B**).

3.3. GH/IGF-1 Signaling Pathway

To investigate the effect of density stress and phenethylamine treatment on the function of the GH/IGF-1 signaling pathway, gene expression levels of GH in the pituitary gland and GHR and IGF-1 in the liver of turbot were analyzed(Figure 4). The results of the density treatment showed that GH mRNA levels were significantly downregulated ($p < 0.05$) in the MD (0.47-fold) and HD groups (0-fold) compared with the LD group. Similar trends were observed for GHR expression in the liver of turbot under different density treatments. In addition, the expression of IGF-1, another key gene located downstream of the GH/IGF-1 signaling pathway, was also significantly lower ($p < 0.05$) in the MD (0.67-fold) and HD (0.63-fold) groups than in the LD group. In phenethylamine treatment, a significant decrease in GH, GHR and IGF-1 gene expression was observed in the LP and HP groups when compared with the CON group ($p < 0.05$). GH expression was almost undetectable in the LP and HP groups.

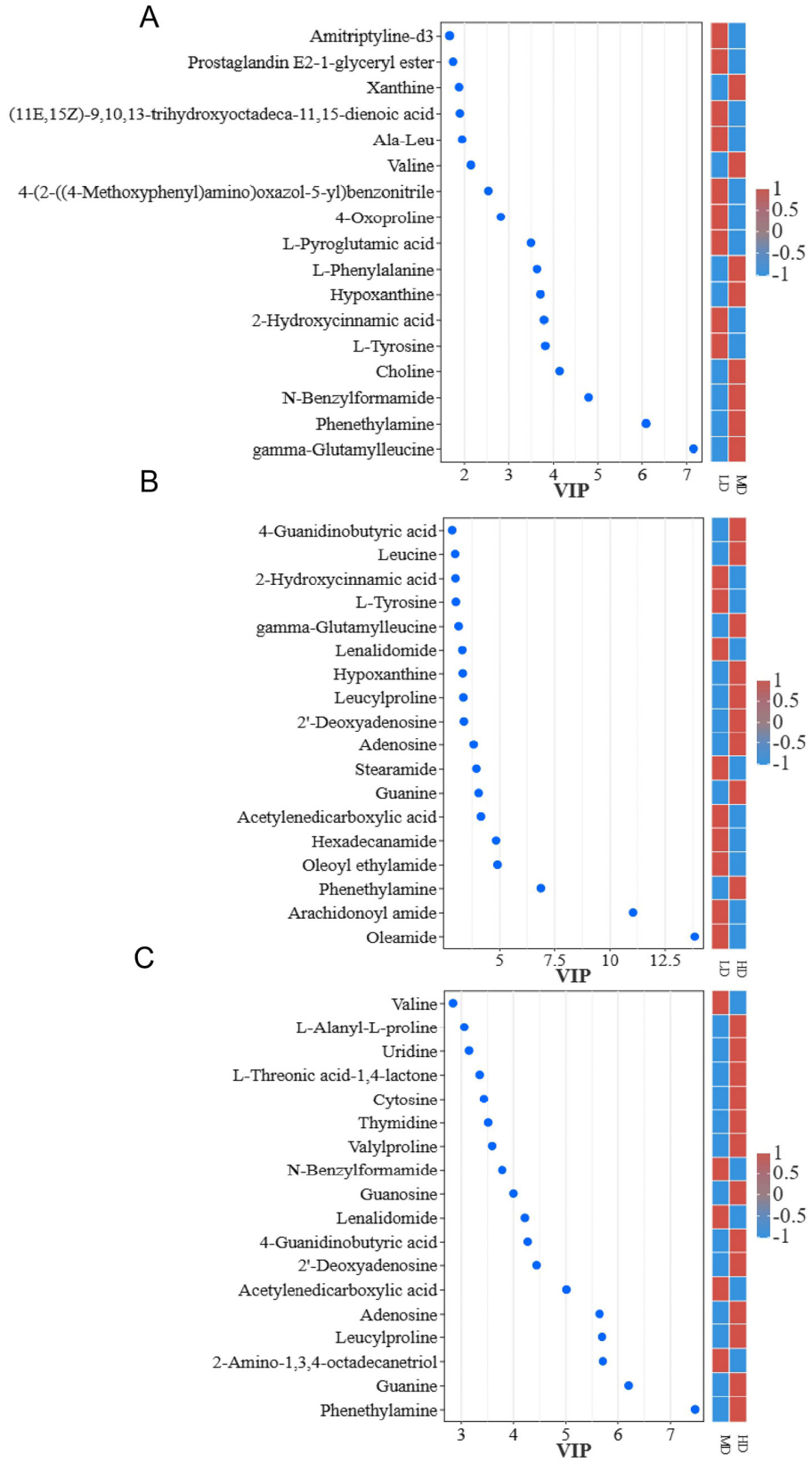

Figure 3. VIP score plot. Variable importance in projection (VIP) scores for the top 18 metabolites. VIP scores are derived from a partial least squares discriminant analysis (PLS-DA) model and colored boxed on the right indicate the relative abundance of the corresponding metabolite in each group. (**A**) LD vs. MD; (**B**) LD vs. HD; (**C**) MD vs. HD.

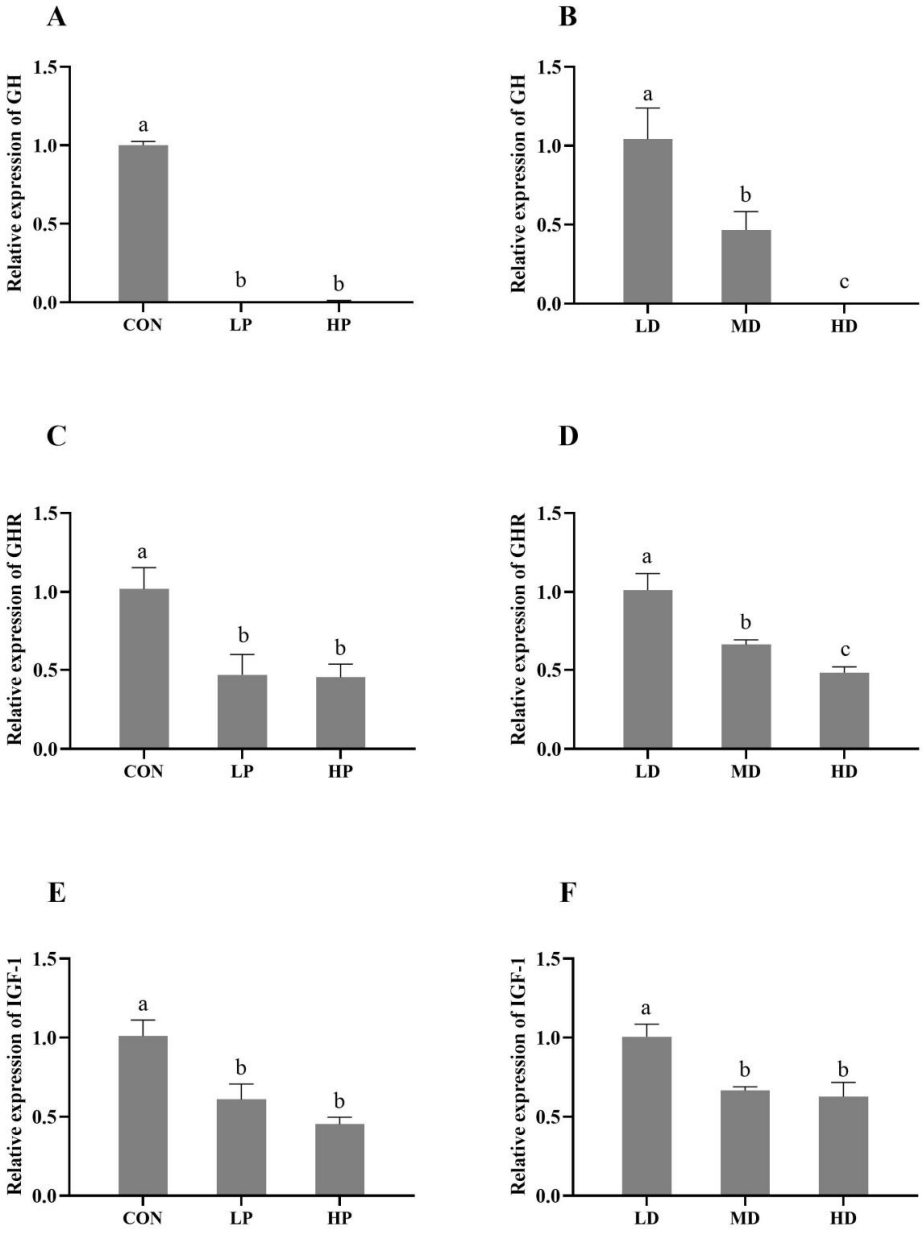

Figure 4. Effects of different stocking densities (LD, MD and HD) and different phenethylamine concentrations (CON, LP and HP) on expression of key genes in GH/IGF-1 signaling pathway in turbot. (**A**) GH relative expression at different phenethylamine concentrations. (**B**) GH relative expression at different stocking densities. (**C**) GHR relative expression at different phenethylamine concentrations. (**D**) GHR relative expression at different stocking densities. (**E**) IGF-1 relative expression at different phenethylamine concentrations. (**F**) IGF-1 relative expression at different stocking densities. The vertical bars represent mean ± S.E. Different lowercase letters indicate significant differences ($p < 0.05$).

3.4. HPI Axis

To assess the effect of density stress and phenethylamine treatment on the HPI axis of turbot, we measured the abundance of key genes (CRH and ACTH) and cortisol levels(Figure 5). The density treatment results revealed that the expression levels of CRH and ACTH genes increased with increasing density, and the HD group treatment exhibited a significantly higher expression than the LD group (1.65 and 1.63 times greater, respectively) ($p < 0.05$). Furthermore, plasma cortisol levels at the end of the HPI axis were significantly higher in the HD group (18.78 ± 0.19 ng/mL) than in the MD group (16.33 ± 0.02 ng/mL)

and LD group (16.17 ± 0.01 ng/mL) ($p < 0.05$). In the phenethylamine treatment, the expression levels of CRH and ACTH genes increased with increasing phenethylamine concentration. Plasma cortisol levels at the end of the HPI axis were significantly higher in the HP group (27.95 ± 0.21 ng/mL) than in the LP group (25.98 ± 0.57 ng/mL) and the CON group (24.64 ± 0.07 ng/mL).

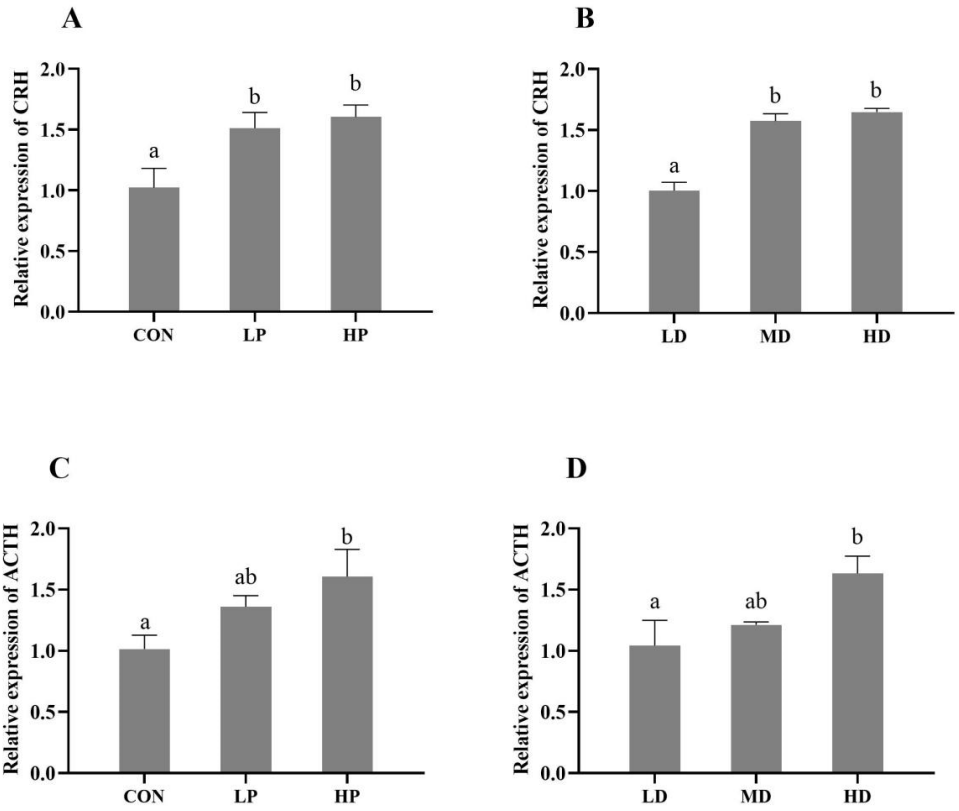

Figure 5. Effects of different stocking densities (LD, MD and HD) and different phenethylamine concentrations (CON, LP and HP) on expression of key genes of HPI axis in turbot. (**A**) CRH relative expression at different phenethylamine concentrations. (**B**) CRH relative expression at different stocking densities. (**C**) ACTH relative expression at different phenethylamine concentrations. (**D**) ACTH relative expression at different stocking densities. The vertical bars represent the mean ± S.E. Different lowercase letters indicate significant differences ($p < 0.05$).

3.5. Physiological Response of Turbot Plasma

The effects of density stress and phenethylamine treatment on T_3, glucose and triglycerides are shown in Figure 6. In the density treatment, plasma glucose, triglyceride and T3 levels were significantly higher in the HD group than in the LD group ($p < 0.05$), while there were no significant differences between the MD and LD groups. In phenethylamine treatment, the levels of plasma glucose, triglycerides and T_3 were significantly higher in the LP and HP groups than in the CON group ($p < 0.05$), while there were no significant differences between the LP and HP groups.

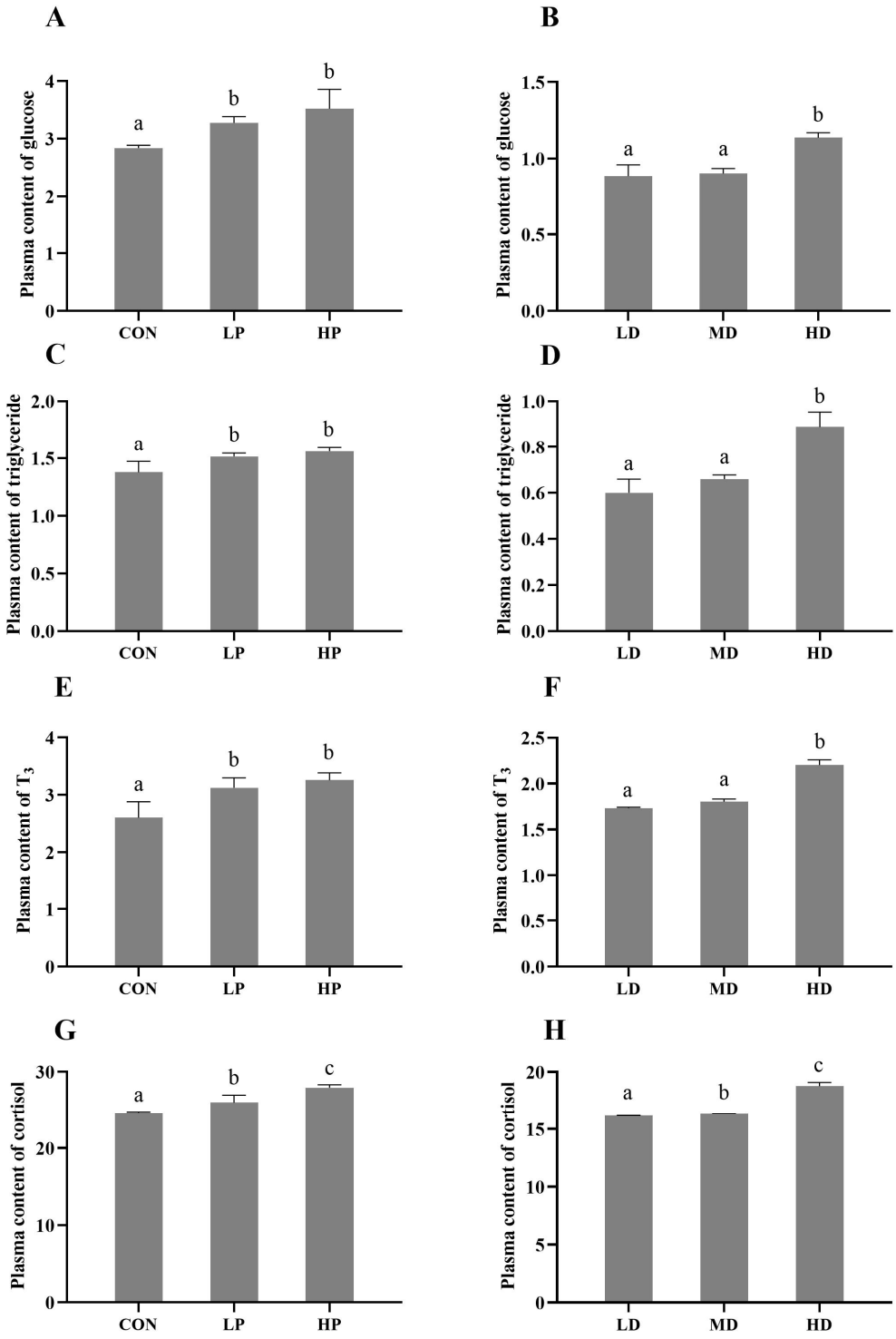

Figure 6. Effects of different stocking densities (LD, MD and HD) and different phenethylamine concentrations (CON, LP and HP) on physiological parameters of turbot plasma. (**A**) Plasma content of glucose at different phenethylamine concentrations. (**B**) Plasma content of glucose at different stocking densities. (**C**) Plasma content of triglyceride at different phenethylamine concentrations. (**D**) Plasma content of triglyceride at different stocking densities. (**E**) Plasma content of T_3 at different phenethylamine concentrations. (**F**) Plasma content of T_3 at different stocking densities. (**G**) Plasma content of cortisol at different phenethylamine concentrations. (**H**) Plasma content of cortisol at different stocking densities. The vertical bars represent the mean ± S.E. Different lowercase letters indicate significant differences ($p < 0.05$).

4. Discussion

In this study, Hexadecanedioic acid, xanthine, phenethylamine, proline and styrene in the metabolite profiles of aquatic environments were significantly different in MD vs. LD, HD vs. MD and HD vs. LD, and the levels increased significantly with increasing density. It is important to note that hexadecanedioic acid and styrene are insoluble in water, while xanthine, phenethylamine and proline are soluble. Solubility determines the spatial extent of the pheromone, and since substances dissolved in water are more likely to diffuse in an aquatic environment, proline, xanthine and phenethylamine are more likely to act as pheromones. Previous studies have shown that substances such as nitrogenous compounds and purines act as aquatic animal pheromones [29,30]. He et al. showed that three common purines (adenosine, inosine and hypoxanthine) released by adult dreissenid mussels (*Mytilopsis sallei*) acted as aggregation pheromones in precise synergistic ratios (1:1.125:3.25) to induce homozygous larval settlement and metamorphosis [29]. Dissanayake et al. confirmed that the behavioral activity contained in the sea lamprey (*Petromzons marinus*) alarm cue resides in the water-soluble fraction of the skin extract, and this water-soluble fraction consisted primarily of creatine (70%), heterocyclic nitrogenous compounds (4.3%) and free amino acids (18.4%), respectively [30]. However, it should be noted that the proline screened in this study was D-proline. Yu et al. showed that L-proline induces an electro-olfactogram (EOG) response in grass carp (*Ctenopharyngodon idellus*) and has a certain degree of usefulness in attracting grass carp [31]. However, the role of D-proline in fish remains poorly understood. In mammals, D-amino acids are often involved in pathophysiological processes and can be used as disease markers [32,33]. Liu et al. demonstrated that D-proline in human peripheral serum may serve as novel biomarker candidates for Alzheimer's disease [34]. Therefore, D-proline was temporarily disregarded as a pheromone in this study. On the other hand, xanthine can be converted to uric acid by the action of xanthine oxidase, and high levels of uric acid induce the release of pro-inflammatory mediators, which leads to inflammatory processes [35]. Baldissera et al. demonstrated that xanthine oxidase activity and uric acid levels increased in the gills of infected silver catfish (*Rhamdia quelen*) with *Streptococcus agalactiae* compared with uninfected silver catfish [36]. Wu et al. showed that hypoxia causes the upregulation of xanthine in rainbow trout (*Oncorhynchus mykiss*) muscle [37]. Therefore, the level of xanthine mainly indicates the health status of an organism and its levels can provide valuable information for the diagnosis and medical treatment of certain metabolic disorders [38]. Phenethylamine is an endogenous amine compound that can play an important biological role in the nervous system as a chemical messenger [39,40]. Low concentrations of phenethylamine produce euphoria, but high concentrations of phenethylamine may form neurotoxic compounds [41]. Phenethylamine can also act as a chemical cue in the external environment to regulate individual animal behavior [42]. Phenethylamine levels have been shown to increase in the urine of stressed mammals [43,44]. In addition, VIP score plots also showed phenethylamine as the most important differential metabolite shared by LD vs. MD, LD vs. HD and MD vs. HD. Thus, phenylethylamine may act as a potential pheromone to transmit information.

The HPI axis plays a crucial role in the response of fish to environmental stresses [45]. When faced with stress, the HPI axis is initially activated, triggering the release of high levels of cortisol in the body as a response to the stressor [46]. Excessive cortisol will induce secondary and tertiary stress responses, resulting in physiological and other functional disorders in fish [47]. Bi et al. discovered that the serum ACTH and cortisol levels of hybrid sturgeon (♀*Acipenser baerii* × ♂*Acipenser schrenckii*) increased with increasing stocking density [48]. Jia et al. observed that a stocking density of 10.8 kg/m^2 for 80 days resulted in a significant increase in plasma cortisol levels in turbot compared with a stocking density of 5.13 kg/m^2 [7]. Additionally, various environmental factors, such as ammonia exposure, nitrate exposure and pathogenic infections, can also upregulate CRH, ACTH genes and plasma cortisol in fish [49–51]. The present study demonstrated that plasma cortisol was significantly higher in the HD group compared with the MD and LD groups, with

increasing density leading to increased expression of CRH and ACTH genes. Under stress, fish release large amounts of cortisol, which increases the metabolism of carbohydrates, fats and proteins and controls the flow of energy in the organism in response to the stressor [52,53]. Liu et al. showed that blood glucose levels were significantly higher in the high stocking density group of turbot than in the other groups [4]. In the present study, plasma glucose and triglycerides were significantly higher in the HD group, which may be due to energy mobilization by the organism to resist the unfavorable external environment, consistent with these results. In teleost fish, the GH/IGF-1 signaling pathway regulates a variety of physiological functions, such as growth, reproduction, immunity and osmoregulation [54–56]. GH levels in fish are positively associated with growth in vivo. Environmental factors such as temperature, salinity, density and other breeding-induced discomforts can cause a decrease in fish IGF-1 levels, often resulting in an impact on fish growth and development [3,57,58]. The present study found that both GH and IGF-1 were significantly downregulated in the HD groups, suggesting the inhibitory effects of high density on turbot growth. Liu et al. studied that the GH concentration in turbot for 120 d in a high stocking density group was significantly decreased [6]. Thyroid hormones (TH) have also been shown to play a crucial regulatory role in fish growth, often working synergistically with other hormones [59,60]. In fish, TH exerts its biological function mainly through the formation of T_3 [61]. Therefore, the study evaluated plasma T_3 levels in turbot. The results indicated that T_3 increased with the increase in density. Ardiansyah and Fotedar found that the T_3 of juvenile barramundi *(Lates calcarifer Bloch*) decreased gradually with the increase in stocking density [62]. This may be attributed to the short duration of density treatment (15 days), during which the fish experience early-stage stress and an elevation in T_3 levels to promote energy metabolism in response to the unfavorable environment. Interestingly, in our study, GH gene expression was almost absent in both HD and HP groups, but IGF-1 gene expression was still present. This may be because the HD and HP groups promoted IGF-1 expression via elevated T_3 acting on the liver. It has been shown that T_3 increases IGF-1 mRNA expression and stimulates the release of IGF-1 [63,64].

The turbot HPI axis was upregulated at high stocking densities, the GH/IGF-1 signaling pathway was downregulated and the concentration of phenethylamine increased with the increase in turbot stocking density. Therefore, the concentration of phenylethylamine was positively correlated with the HPI axis and negatively correlated with the GH/IGF-1 signaling pathway. Subsequently, phenylethylamine acute treatment experiments were designed to verify the effects of phenylethylamine on the HPI axis and GH/IGF-1 signaling pathway. Similarly, plasma cortisol was significantly higher in the HP group compared with the LP and CON groups, with increasing phenylethylamine concentrations leading to increased expression levels of CRH and ACTH genes. This suggests that stress was produced in turbot under phenylethylamine acute treatment. Bredy and Barad showed that phenethylamine can act as a pheromone to communicate information about fear or threats [65]. Ferrero et al. found that phenethylamine induced strong avoidance responses in rodent and herbivore species [66]. Imre et al. showed that sea lamprey (*Petromyzon marinus*) also showed a strong avoidance response to phenethylamine [67]. The present study also observed that GH and IGF-1 were significantly downregulated in the HP group, and some physiological parameters (T_3, glucose, triglycerides) were upregulated in the HP group. These findings indicated that phenethylamine accumulation in the RAS can negatively affect fish physiology and growth. Phenethylamine concentration was strongly correlated with stocking density, and the effects of phenethylamine treatment and density treatment on the HPI axis, GH/IGF-1 signaling pathway and key physiological indicators (cortisol, T3, glucose, triglycerides) were highly similar. In the present study, phenethylamine was detected at LD, MD and HD, but only the turbot in the HD group produced significant stress and growth inhibition. This suggests that the effects of phenethylamine (harmful or beneficial) are dose dependent under specific conditions. In the phenethylamine treatment experiment, even the LP group (10^{-7} mol/L) had a significant negative effect on turbot, which indicates that phenethylamine has a trace effect. However, there

may be differences between the aquaculture experimental system and the actual production system. Therefore, future experiments will be conducted to quantify phenethylamine in an actual production system of turbot and to verify the long-term effects of phenethylamine on turbot.

5. Conclusions

Phenylethylamine is a compound that can be synthesized, secreted, released and perceived by turbot. Phenylethylamine concentration is closely related to stocking density, and phenylethylamine accumulated in water under high stocking density conditions may act as a pheromone to signal crowding stress. In the present study, phenethylamine was detected at LD, MD and HD, but only the turbot in the HD group produced significant stress and growth inhibition, suggesting that the effects of phenethylamine are dose dependent. Even 10^{-7} mol/L of phenylethylamine caused upregulation of the key genes CRH and ACTH on the HPI axis, downregulation of the key genes GH and IGF-1 on the GH/IGF-1 signaling pathway, as well as disorders of plasma cortisol, glucose, triglyceride and T_3 in turbot. Therefore, phenethylamine has trace effects. These findings provide new insights for further exploration of density stress mechanisms.

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Data Availability Statement: The data used during the current study are available upon reasonable request.

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