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Early-life conditioning induces different transcriptional but not microbial responses to later life stress in diploid and triploid trout

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Abstract

Early-life exposure to stressors can alter responses to later-life stress in either positive or negative ways, potentially mediated by multiple mechanisms, including changes in transcriptomic pathways and/or host-associated microbial communities. We investigated whether early-life stress conditioning during embryonic development influences later-life stress responses and how it differs between ploidies. Diploid and triploid rainbow trout (*Oncorhynchus mykiss*) embryos were exposed to a controlled cold shock (early-life conditioning) and then challenged with acute and chronic crowding stressors 15 weeks later (juvenile stage). Long-term stress response was assessed using transcriptome and microbiota profiling. We found that conditioned fish exposed to acute and chronic crowding stress showed a pronounced reduction in transcriptional stress response compared with unconditioned fish and responses were ploidy dependent. In contrast, microbiota analysis revealed that early-life conditioning and crowding stress altered microbial community structure independently, but that early-life conditioning did not influence subsequent microbiota response to later life stress. Overall, our results show that early-life conditioning induced long lasting effects on the later life stress responses, although the transcriptome and gut microbiota responded in different ways.

1. Introduction

Stress responses are physiological and behavioural reactions triggered by physical or psychological stimuli, including many environmental factors, that disrupt homeostasis (Chu et al. 2024). The Stress response has been extensively studied in teleosts (Gorissen & Flik. 2016; Schreck & Tort. 2016; Petitjean et al. 2019), particularly the cascade of hormonal events regulated by the hypothalamus–pituitary–interrenal (HPI) axis, which mediates subsequent osmoregulatory and metabolic adjustments (Alsop & Vijayan. 2009; Tsalafouta et al. 2014; Amano et al. 2022; Zheng et al. 2024). The effects of a specific stressor depend on its timing, duration, magnitude, and nature (Schulte. 2014; Gunderson et al. 2016). Traditionally, stress has been considered detrimental to organism's performance (Conte. 2004; Kassahn et al. 2009; Asres & Amha. 2014; Sadoul & Vijayan. 2016); however, recent studies suggest that mild stressors applied early in life can act as a "conditioning" event, potentially programming and altering the organism's response to stressful events in later stages via stress memory and developmental plasticity (Moghadam et al. 2015; Moghadam et al. 2017; Robinson et al. 2019). Understanding these differences and the underlying molecular mechanisms of early-life conditioning could be used to enhance productivity and improve stress tolerance and disease resilience in livestock and aquaculture systems.

Early developmental stages, such as embryogenesis and larval development, represent critical windows of heightened plasticity (Vagner et al. 2019). Developmental and epigenetic programming occurring during early developmental stages more sensitive to environmental stressors including thermal fluctuations, hypoxia, and pathogen exposure (Jonsson & Jonsson. 2019; Vagner et al. 2019; Mäkinen et al. 2023). Environmental perturbations experienced during early development can induce long-lasting physiological and metabolic changes, including negative effects on growth, behaviour (Derks et al. 2017), disease resistance, and overall fitness later in life (Barrera. 2017; Jonsson & Jonsson. 2019; Burggren. 2020; Buchanan et al. 2022). However, recent studies demonstrate that environmental perturbations experienced during early life can also trigger adaptive responses through developmental plasticity (Jonsson & Jonsson. 2019; Earhart et al. 2022; Liu et al. 2022a). Overall, the disruptive or conditioning effects of early-life stress likely depend on the developmental stage, type, nature and duration of the stressor (Moghadam et al. 2015; Jonsson & Jonsson. 2019; Robinson et al. 2019; Vagner et al. 2019).

Aquaculture is one of the fastest-growing sectors in global food production (FAO. 2024). The increasing demand for fish and seafood products has driven a rapid intensification of commercial aquaculture systems worldwide (Hanke et al. 2020; FAO. 2024). However, this intensification exposes farmed fish to various environmental and husbandry-related stressors, including fluctuations in temperature (Naz et al. 2023), low oxygen (Yang et al. 2022), repeated handling (Eissa & Wang. 2016), and high stocking densities (Liu et al. 2017). These stressors can affect fish behaviour (Rodriguez-Barreto et al. 2019), growth (Sadoul & Vijayan. 2016), immune function (Ellison et al. 2018), microbiota (Uren Webster et al. 2021; Zhou et al. 2022), health and overall welfare of farmed fish (Conte. 2004; Varsamos et al. 2006). As a result, there is a need to increase resilience to these stressors, and early-life conditioning may be a promising strategy to achieve this (Moghadam et al. 2015).

The cross talk between the host immune system and the microbiota plays a crucial role in stress responsiveness (Houwenhuyse et al. 2021; Czech et al. 2022), adaptive plasticity (Alberdi et al. 2016) and maintenance of physiological homeostasis (Nicholson et al. 2012). Host-associated microbiota, especially the bacteria associated with the gut, play an important role in the development and maturation of the host immune system (Kelly & Salinas. 2017), nutrient uptake, metabolism, and resistance to pathogens (Nicholson et al. 2012). In vertebrates, early-life developmental stages are crucial for microbiota colonisation and for the development and maturation of the immune system (Donald & Finlay. 2023). Early colonized microbiota influences the establishment, succession, and stability of microbial communities (Sprockett et al. 2018; Ortega et al. 2021). Through priority effects, these early colonizers drive persistent changes in the communities' structure and functional capacity (Debray et al. 2022; Jones et al. 2024). Environmental stressors can disrupt early colonizers and alter microbial succession (Reemst et al. 2022). Such early-life microbiota perturbations may have long-lasting impact on the host physiology and stress regulation (Foster et al. 2017; Reemst et al. 2022).

In addition to influencing early microbiome colonization, stress during critical stages of host development also modifies gene regulatory networks through persistent transcriptional and epigenetic changes, resulting in long-lasting alterations in gene expression that influence immunological, metabolic, and neuroendocrine development (Vindas et al. 2016; Girgenti et al. 2021; Madaro et al. 2023; Norloff et al. 2026). Later in life, these molecular alterations may modify the host's sensitivity and adaptive reactions to stress (Sarropoulou et al. 2016; Robinson et al. 2019). Further, studies have proven that microbiota can also influence the host gene expression under stressful conditions, and it is likely that complex bidirectional interactions occur between microbiota and host regulatory pathways (Su et al. 2021; Liu et al. 2023; Norloff et al. 2026).

To understand the relationship between early-life conditioning and the stress response, we examined the response of rainbow trout (*Oncorhynchus mykiss*), one of the most important cold-water aquaculture species worldwide (Sun et al., 2023; FAO, 2024), to two stressors. We investigated the transcriptome and microbiota responses of juvenile rainbow trout to crowding stress (acute and chronic) applied after receiving an early-life stressor (embryonic cold shock and

air exposure, hereafter referred to as “early-life conditioning”), to test the hypothesis that early-life conditioning persistently alters transcriptional stress response as well as microbiota structure and composition. We compared the responses of triploid and diploid trout, as the use of triploids is common in aquaculture due to their sterility and often enhanced aquacultural traits, such as growth performance and meat quality (Piferrer et al. 2009; Keyvanshokooh. 2025), but can also be more susceptible to crowding stress than diploid (Mahmoudi et al. 2025).

2. Methods

2.1 Fish husbandry, early-life conditioning, crowding stress exposure and sample collection

A total of 1,826 eyed rainbow trout eggs, half diploid and half pressure induced triploid were obtained from a commercial hatchery in May 2023. Eggs were randomly allocated to eight identical trays within a single vertical incubator at the Centre for Sustainable Aquatic Research (CSAR), Swansea University (Swansea, UK), with diploid and triploid eggs kept separately. Two trays from each ploidy were randomly marked for treatment (cold shock), the remaining two were designated controls. Each of the eight trays (two diploid cold shock, two diploid control, two triploid cold shock, two triploid control) were then randomly placed in the incubator to minimise potential tank effects. Temperature, pH and dissolved oxygen were measured daily, while ammonia, nitrite, nitrate and salinity were measured weekly.

The cold shock treatment was performed as follows: the day after their arrival, all cold shock trays were placed in a bath of iced water (0–2°C) for one-minute, this was followed by a one-minute air exposure (cold shock treatment) following the protocol outlined in Moghadam et al (2017). The control trays were submerged in ambient water from the vertical incubator for one minute instead of the iced water bath followed by a one-minute air exposure, this was to mitigate any potential effects of all other variables except for water temperature (Fig. 1). After the treatment, the egg trays were returned to their original position in the vertical incubator. This was repeated once more one week after the first treatment, just before the start of hatching. Eggs were kept in the vertical incubator until approximately 75% of them hatched (12 days after arrival), they were then transferred to eight alevin troughs, before being moved into 115 L tanks when they reached 1-1.5 g. Fish were reared in these tanks for 105 days (15 weeks) before the crowding stress experiment began.

For the crowding stress trials, fish from each tank were divided into three groups (24 total, each with ~ 15 fish), the three tanks for each treatment were assigned to a different stress group, namely acute, chronic, or control (no stress) (Fig. 1). The stress trial consisted of lowering the water level by half for 1 hour for 3 days for the acute stress group and 14 days for the chronic stress, the water volume was not manipulated in any way for the control group. After the crowding stress trial, five fish per tank, with a total of ten fish per group, were randomly chosen and sacrificed following Home Office guidelines (Schedule 1). Length and weight were collected for each fish, alongside intestine tissue and hind gut content (faeces), for transcriptome and microbiota analysis. Intestine samples were preserved in RNAlater and stored at − 80°C, faeces samples were immediately stored at -80°C without any buffer until further analysis.

2.2 RNA extraction and sequencing

Total RNA was extracted from the frozen intestine samples (posterior intestine) using AllPrep DNA/RNA Mini Kit (Qiagen, Hilden, Germany) and Direct-zol™ RNA MiniPrep (Zymoresearch, CA, USA) following the manufacturer’s instructions. Extracted RNA samples were shipped to Functional Genomics Center Zurich (FGCZ) University in Zürich, Switzerland on dry ice. The quality of the isolated RNA was determined with a Fragment Analyzer (Agilent, Santa Clara, CA, USA). All samples had RIN values ≥ 5 (minimum − 5.3; median − 8; maximum − 10)]. The Illumina Stranded mRNA Prep, Ligation (Illumina, Inc, CA, USA) was used in the succeeding steps. Briefly, total RNA samples (100–200 ng) were poly A enriched using oligo(dT) magnetic beads. Purified mRNA was fragmented and converted into double-stranded cDNA through sequential first- and second-strand synthesis. The cDNA samples were adenylated before ligation of RNA index anchor adapters. Fragments with adapters on both ends were selectively enriched by PCR using unique dual-index (UDI) primers for multiplexing. The quality and quantity of the enriched libraries were validated using the Fragment Analyzer (Agilent, Santa Clara, CA, USA). The product is a smear with an average fragment length of approximately 300–400 bp. The libraries were normalized to 10nM in Tris-Cl 10 mM, pH8.5 with 0.1% Tween 20. The Novaseq X Plus (Illumina, Inc, CA, USA) was used for cluster generation and sequencing according to standard protocol. Sequencing configuration was paired-end 150 bp.

2.3 DNA extraction and sequencing

DNA was extracted from the faeces samples using DNeasy PowerLyzer PowerSoil Kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions. The extracted DNA was amplified using bacterial primers targeting the 16S gene V4 region (515F, 806R) (Apprill et al. 2015; Parada et al. 2016). All PCR reactions consisted of a mix of 12.5 µl of Platinum™ II Hot-Start PCR Master Mix (Thermo Fisher Scientific, USA), 0.5 µl of each primer (1µM), 9 µl of water and 2.5 µl of DNA in a total volume of 25 µl using a Bio-Rad T100 thermal cycler, with the following conditions: 3 minutes at 95°C followed by 28 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds and a final 5 minutes at 72°C. Index PCR (8 cycles) was then performed with the first PCR product as a template using Nextera XT Index primers (Illumina Inc., USA), with the above conditions. Amplified PCR products were visualised using gel electrophoresis, and concentrations were normalised based on band intensity before purification with Ampure XP beads (Beckman Coulter Genomics, USA). Paired end (300 bp) sequencing of the prepared amplicon libraries was performed at the Institute of Life Science, Swansea University (Swansea, UK) using the Illumina MiSeq® platform with a MiSeq® reagent kit (Illumina Inc.).

2.4 Bioinformatic and statistical analysis

Transcriptome analysis

The quality of raw reads was assessed using *FASTQC* v0.11.9 (Andrews et al. 2010) and *MultiQC* v1.14 (Ewels et al. 2016). Subsequently, *Fastp* v0.19.10 (Chen et al. 2018) were employed to filter out low-quality reads (Phred score < 30), short reads and adapter sequences. The filtered reads were used for downstream analysis. Reference genomes and annotations of the rainbow trout (USDA_OmykA_1.1) for mapping and indexing were downloaded from the National Centre of Biotechnology Information (https://www.ncbi.nlm.nih.gov). *HISAT2 v2.2.1* (Kim et al. 2015) was used for genome indexing and mapping of the filtered reads with default parameters. The aligned reads were annotated using *featureCounts v1.6.3* (Liao et al. 2014) to obtain the read counts. Differential expression of the genes across the treatment groups was analysed by *DESeq2* package (Love et al. 2014). Read counts were filtered to keep only genes with > 10 reads in each sample using the filtering step in *DESeq2* package. The diploid and triploid datasets were treated separately to identify differentially expressed genes (DEGs) to examine the transcriptomic differences between ploidies. We fitted multifactor DESeq2 model [Counts ~ Weight + Early-life conditioning + Crowding stress (acute and chronic) + Early-life conditioning: Crowding stress (acute and chronic)] to identify DEGs associated with early-life conditioning, crowding stress (acute and chronic stress), and their interaction, in order to examine the effect of early-life conditioning on the later life stress responses. Transcripts with $|\text{Log}_2$ fold change$| \geq 1$ and an adjusted *p*-value of < 0.05 (Benjamini–Hochberg multiple test correction method) were considered significantly differentially expressed. Gene ontology (GO) enrichment analyses of DEGs were performed using *clusterProfiler* (Wu et al. 2021), to identify significantly enriched biological processes (BP), molecular functions (MF), and cellular components (CC) of particular stress treatment groups. All statistical analyses were performed in R version 4.2.1 (RCoreTeam. 2021) using RStudio version 2022.12.0, and all figures were generated with the *ggplot2* package (Wickham. 2011).

Microbiota analysis

Raw amplicon paired-end reads were quality checked using *FASTQC* v0.11.9 (Andrews et al. 2010) and *MultiQC* v1.14 (Ewels et al. 2016) then imported into *QIIME2* v2022.11 (Bolyen et al. 2019). The *cutadapt* plugin in *QIIME2* was used to trim the adaptor sequences before reads were demultiplexed and denoised using *DADA2* (Callahan et al. 2016; Bolyen et al. 2019). Identification and taxonomic classification of Amplicon Sequence Variants (ASVs) were performed with *QIIME2's* feature-classifier function using a naïve Bayes classifier trained on the SILVA reference database (v138) (Quast et al. 2012). The phylogenetic tree, taxonomy and ASVs table were imported in to R as a phyloseq object (McMurdie & Holmes. 2013) using *qiime2R* scripts (Bisanz. 2024) for further analysis. ASVs were filtered by removing those assigned to cyanobacteria and mitochondria. To account for variation in sequencing depth and prepare the data for downstream analysis, the data were rarefied to 69,400 reads per sample.

To evaluate the effect of stress treatment on microbial alpha diversity between treatment groups and ploidy, Shannon diversity (community diversity), Chao1 richness (species richness) and Simpson diversity (evenness of the community) were calculated using the *phyloseq* package. Alpha diversity indices were analysed in R using the *MuMIn* (Barton. 2012), *lme4* (Bates et al. 2015) and *car* (Fox et al. 2012) packages. For each alpha diversity index (Shannon, Simpson, and Chao1), a linear mixed effects model was fitted including fixed effects of fish weight, ploidy, early-life conditioning (cold shock), crowding stress (acute and chronic), their interaction (cold shock × crowding stress), and tank as a random factor. Model selection was performed using maximum likelihood (REML = FALSE). The interaction term (cold shock × crowding stress) was evaluated first and removed when statistically not significant. Weight was then assessed and excluded if it did not improve model fit. Akaike's Information Criterion (AIC) and the Bayesian Information Criterion (BIC) were subsequently used to compare candidate models and identify the simple model. Models with $\Delta\text{AIC} \leq 2$ were considered to have similar support. Fish weight from all final models, and cold shock from the Chao1 model was excluded, because their inclusion did not improve model fit based on comparisons of the AIC and BIC. The final selected models were refitted using restricted maximum likelihood (REML = TRUE). Finally, model assumptions were evaluated using the *performance* package (Lüdecke et al. 2021).

To characterise the microbial community structure, Bray–Curtis dissimilarity was used to evaluate variation in beta diversity using the *vegan* package (Oksanen et al. 2013). Non-metric multidimensional scaling (NMDS) ordination was then performed to visualise the beta diversity structure using the *phyloseq* package. Multivariate statistical analysis of the gut microbial community was performed by PERMANOVA using the *adonis2* function in the *vegan* package with 10000 permutations, this model included fish weight, ploidy, early-life conditioning, crowding stress as fixed effects and tank was included in the model using the *strata* function.

The relative abundance and taxon differences between treatment groups and ploidy at the phylum and family level were visualised using the *microViz* (Barnett et al. 2021) and *microbiome* (Lahti & Shetty. 2018) packages. Finally, the *DESeq2* (Love et al. 2014) package was used to identify the differentially abundant ASVs between conditioned and unconditioned fish within the same crowding stress treatment using the non-rarefied data (McMurdie & Holmes. 2013).

Integrated analysis of gut microbiota and host gene expression

To study host-microbiota interactions in the conditioned fish, an integrated microbiota-transcriptome analysis was performed. Separate analyses were performed for diploid and triploid fish that experienced early-life conditioning and exposed to later-life crowding stress. DEGs and their normalised expression values were obtained from the transcriptome analysis, while the top 20 most abundant ASVs were selected from the microbiota dataset and subjected to centred log-ratio transformation (CLR). Then, pairwise associations between microbial ASVs abundance and host genes expression were evaluated using univariable Spearman rank correlations (*stats* package), with false discovery rate correction (Benjamini–Hochberg) applied to identify significant ASV-DEGs relationships.

3. Results

3.1. Summary of sequencing data

A total of 4827.4 M raw reads were obtained from 102 RNA-seq libraries (from 60 diploid and 60 triploid samples, 5 diploid and 13 triploid samples were removed due to low RNA quality). After adapter trimming and quality filtering (> Q30), 4487.1 M reads were retained. The average mapping rate of clean reads was 93.31% in diploid and 92.78% in triploid groups. The detailed information can be found in the Supplementary data.

Sequencing of the amplicon libraries of 16S rRNA gene, yielded 16.85 M reads with an average 140,452 reads per sample. Reads were rarefied to 69400 reads per sample, which resulted in removal of one sample from the diploid group (4D1) (see Supplementary data).

3.2 Early-life conditioning and crowding stress induced distinct transcriptome responses

Principal component analyses (PCA) of gene expression profiles revealed no distinct clustering between stress treatment groups in either diploid or triploid fish (**Figure S1**).

Early-life conditioning (cold shock) induced persistent transcriptional changes in both ploidy groups. In diploid fish, 661 DEGs (215 upregulated and 446 downregulated genes) were identified 17 weeks after exposure compared with controls. In contrast, only 174 DEGs (144 upregulated and 30 downregulated) were detected in triploid fish (Fig. 2 **and Figure S2**), indicating fewer long-term transcriptional changes in triploids compared with diploids. Gene ontology analysis revealed that diploid fish DEGs were associated with enrichment of DNA processing and repair, protease inhibition and modification, cilia- and microtubule-related processes, extracellular matrix organisation and collagen metabolism, most of these GO terms were related to active tissue remodelling and structural maintenance processes (**Figure S3**). Triploid DEGs were associated with enrichment of GO terms related to protein folding, chaperone activity, chromosome organisation, phosphocreatine metabolism and creatine kinase activity (**Figure S4**).

To assess the effects of crowding stress on transcriptional response, differential gene expression analysis was performed in diploid and triploid unconditioned fish under acute and chronic stress conditions. In diploid fish, acute crowding stress resulted in 1,448 DEGs compared with controls, whereas chronic crowding stress resulted in 989 DEGs (Fig. 2A, Fig. 3A **and** Fig. 3B). In triploid fish, 827 DEGs were identified under acute stress and 1,008 DEGs under chronic stress (Fig. 2B, Fig. 3C **and** Fig. 3D). Overall, diploid fish exhibited a greater transcriptional related response to acute crowding stress, as indicated by the higher number of DEGs compared with triploid fish.

Gene ontology analysis revealed that in diploid fish exposed to acute crowding stress, the upregulated genes were significantly enriched in immune related processes, particularly T-cell activation and adaptive immune responses including chemotaxis and chemokine receptor activities (**Figure S5A)**. Whereas downregulated genes were enriched in GO terms involved in extracellular matrix organisation, amino acid and fatty acid biosynthesis and metabolism (**Figure S5B)**. Furthermore, the GO enrichment analysis based on the chronic stress upregulated genes revealed significant enrichment of energy metabolism related GO terms including mitochondrial oxidative phosphorylation and ATP generating pathways (**Figure S4A)**. Downregulated genes were enriched for the processes related to cellular motility and tissue structural maintenance (**Figure S6B**).

In triploid fish exposed to acute crowding stress, the upregulated genes exhibited significant enrichment of immune-related pathways, adaptive immune responses, and lipid metabolism (**Figure S7A**). Only a single significantly enriched GO term was identified among the downregulated genes. Under chronic crowding stress, upregulated genes significantly enriched for terms related to ribosome biogenesis and protein synthesis, mitochondrial energy production, lipid metabolism, RNA processing and splicing (**Figure S7B**). Downregulated genes were enriched in the smoothened signalling pathway and G protein-coupled receptor binding, indicating reduced signalling activity under chronic stress.

3.3 Identification of candidate stress biomarkers shared across ploidies and stress treatments

To identify genes that were consistently differentially expressed across all stress treatments compared to controls, we conducted intersection analysis of DEGs including conditioned and unconditioned fish exposed to acute and chronic crowding stress in both diploid and triploid fish. Nine genes were found to be shared across six of the ten treatment groups in both ploidies (Table 1 **and Figure S8**). Notably, three genes *LOC110524408* (DnaJ (Hsp40) homolog, subfamily C, member 19), *mntb* (MAX network transcriptional repressor b) and *LOC118965590* were shared in seven treatment groups (Table 1).

Table 1
List of common genes associated with six out of ten treatment conditions in both ploidies.

Gene	Gene Name
atp2a1l	ATPase sarcoplasmic/endoplasmic reticulum Ca2 + transporting 1, like
ddx6	DEAD (Asp-Glu-Ala-Asp) box helicase 6
LOC110518775	ensconsin
LOC110524408	DnaJ (Hsp40) homolog, subfamily C, member 19
LOC110525252	serine-rich coiled-coil domain-containing protein 1
LOC110532860	constitutive coactivator of PPAR-gamma-like protein 2
LOC110533276	uncharacterized LOC110533276
LOC118965590	uncharacterized LOC118965590
mntb	MAX network transcriptional repressor b
Figures:	

Table S1
Differentially abundant ASVs in diploid conditioned fish compared with unconditioned fish under acute stress.

log2FoldChange	lfcSE	stat	padj	Phylum	Class	Order	Family	Genus
-23,8765175	2,95780143	-8,07238689	4,0742E-13	*Firmicutes*	*Bacilli*	*Lactobacillales*	*Lactobacillaceae*	*Lactobacillus*
-22,4691359	2,95984189	-7,5913298	9,3567E-12	*Firmicutes*	*Bacilli*	*Lactobacillales*	*Lactobacillaceae*	*Lactobacillus*
20,9319453	2,95777426	7,07692457	2,9036E-10	*Actinobacteriota*	*Actinobacteria*	*Micrococcales*	*Microbacteriaceae*	
7,25747708	1,55842637	4,65692652	0,00047423	*Firmicutes*	*Bacilli*	*Lactobacillales*	*Lactobacillaceae*	*Lactobacillus*

Table S2
Differentially abundant ASVs in diploid conditioned fish compared with unconditioned fish under chronic stress.

log2FoldChange	lfcSE	stat	padj	Phylum	Class	Order	Family	Genus
-24,1333132	2,35734503	-10,2374972	6,5587E-22	*Firmicutes*	*Bacilli*	*Lactobacillales*	*Lactobacillaceae*	*Lactob*
26,5150367	2,96301883	8,94865615	8,762E-17	*Proteobacteria*	*Gammaproteobacteria*	*Enterobacterales*	*Enterobacteriaceae*	
25,8831179	2,9634387	8,73414991	3,9852E-16	*Proteobacteria*	*Gammaproteobacteria*	*Enterobacterales*	*Enterobacteriaceae*	
25,7331334	2,96348227	8,68341063	4,6761E-16	*Proteobacteria*	*Gammaproteobacteria*	*Enterobacterales*	*Enterobacteriaceae*	
4,55429937	1,0980492	4,14762778	0,00327204	*Proteobacteria*	*Gammaproteobacteria*	*Enterobacterales*	*Enterobacteriaceae*	

Table S3
Significant correlations between *Lactobacillus* ASVs and host gene expression in diploid conditioned fish identified by Spearman correlation anal

ASV	Family	Genus	Species	Gene	Spearman_rho	pvalue
79bd328dc25ff772360397901a41aa7d	*Lactobacillaceae*	*Lactobacillus*		*slitrk4*	-0,891894584	1,51554E-06
5be530b4edbf49e267793b3090f9c5bd	*Lactobacillaceae*	*Lactobacillus*		*slitrk4*	-0,906636643	5,27443E-07
6ef6eecaf361bae67ecd8bd32c29b204	*Lactobacillaceae*	*Lactobacillus*	*Lactobacillus_secaliphilus*	*slitrk4*	-0,891894584	1,51554E-06
fec8c0e8e53044511d54908761d4ad22	*Lactobacillaceae*	*Lactobacillus*		*slitrk4*	-0,901722624	7,63637E-07
9e25a413d3b98d697a5bd34961a2c5d5	*Lactobacillaceae*	*Lactobacillus*	*Lactobacillus_secaliphilus*	*slitrk4*	-0,884523554	2,43107E-06
5d2356d8798c11abda797ab361d25f33	*Lactobacillaceae*	*Lactobacillus*	*Lactobacillus_amylovorus*	*slitrk4*	-0,869781495	5,72563E-06
b41ee1a4c620c14db36b3b7f2aeac427	*Lactobacillaceae*	*Lactobacillus*	*Lactobacillus_amylovorus*	*slitrk4*	-0,850125416	1,54807E-05
6ef6eecaf361bae67ecd8bd32c29b204	*Lactobacillaceae*	*Lactobacillus*	*Lactobacillus_secaliphilus*	*LOC110531996*	-0,850490196	1,52173E-05

Supplementary figures:

3.4 Early-life conditioning modified the transcriptional related stress response to later life stress

To understand whether conditioning during the embryonic stage could influence resilience to later life crowding stress, we compared the gene expression patterns in conditioned and unconditioned fish exposed to acute or chronic crowding stress during the juvenile stage. Early-life conditioning was associated with a reduced magnitude of the transcriptional response to later life crowding stress exposure (Diploid - acute stress: 496 DEGs = 65.7%, chronic stress: 539 DEGs = 45.5%; Triploid - acute stress: 509 DEGs = 49.5%, chronic stress: 692 DEGs = 31.3%). (Fig. 2 **and** Fig. 4). The conditioning induced a reduction in the magnitude of the transcriptional response which were ploidy dependent, and both ploidies exhibited distinct responses to acute and chronic stress.

Gene ontology analysis for the conditioned diploid fish which were exposed to an acute stress at the juvenile stage revealed GO terms among downregulated genes related to kinase activity, glucose and carbohydrate metabolism, cytoskeletal remodelling and microtubule dynamics (**Figure S9A**). In conditioned diploid fish exposed to chronic stress, terms related to ribosome biogenesis and translation, mitochondrial respiration and ATP production, iron homeostasis were enriched among upregulated genes (**Figure S9B**). On the other hand, enriched GO terms in conditioned triploid fish that were exposed to acute stress were related to aromatic amino acid metabolism, vesicle targeting and TORC1 signalling pathways. Whereas in chronic stress ribosome biogenesis and translation, RNA processing and splicing, cytoskeletal remodelling were the enriched GO terms among upregulated genes (**Figure S10**).

3.5 Effects of ploidy, early-life conditioning, and crowding stress on gut microbial alpha and beta diversity

We tested whether acute or chronic crowding stress induced microbiota disruption in diploid and triploid fish, and whether early-life conditioning improves the recovery from crowding stress induced disruption. Fish weight and the interaction between cold shock and crowding stress were excluded from the final model because they were not statistically significant and did not improve model fit. Shannon diversity was significantly influenced by ploidy ($t_{(19.13)} = -5.72$, $P < 0.001$), early-life conditioning ($t_{(19.13)} = -2.46$, $P = 0.024$), whereas acute stress ($t_{(18.95)} = 2.04$, $P = 0.055$), and chronic stress ($t_{(19.22)} = -1.23$, $P = 0.23$) showed no significant effects (Fig. 5). Likewise, Simpson diversity was significantly influenced by ploidy ($t_{(19.13)} = -5.14$, $P < 0.001$) and early-life conditioning ($t_{(19.13)} = -2.54$, $P = 0.02$), whereas acute stress ($t_{(18.97)} = 1.44$, $P = 0.16$), and chronic stress ($t_{(19.20)} = -1.3$, $P = 0.21$) showed no significant effects. Finally, Chao1 richness was significantly influenced by ploidy ($t_{(20.18)} = -6.16$, $P < 0.001$) and acute stress ($t_{(19.98)} = 3.36$, $P = 0.003$), whereas chronic stress ($t_{(20.27)} = -0.98$, $P = 0.33$) showed no significant effects. In addition to the effects of individual stressors, we also evaluated the overall effect of crowding stress on alpha diversity. Crowding stress significantly affected all three alpha diversity metrics. Shannon diversity ($F_{(2, 19.13)} = 5.44$, $P = 0.013$) and Simpson diversity ($F_{(2, 19.13)} = 3.74$, $P = 0.043$), was significantly affected with crowding stress and Chao1 richness showed the strongest response to crowding stress ($F_{(2, 20.17)} = 10.35$, $P < 0.001$). Overall, triploid fish displayed lower alpha diversity across metrics, including Shannon (estimate = − 0.82), Simpson diversity (estimate = − 0.27) and Chao1 richness (estimate = − 5.11) compared to diploids (Fig. 5).

NMDS analysis based on Bray–Curtis dissimilarity revealed no clear structuring in the microbial community across treatments (**Figure S11**). Further, PERMANOVA analysis revealed that microbial community composition was not significantly affected by fish weight ($R^2 = 0.006$, $P = 0.66$), ploidy ($R^2 = 0.23$, $P = 0.45$), early-life conditioning (Cold shock; $R^2 = 0.06$, $P = 0.40$), and acute or chronic stress ($R^2 = 0.06$, $P = 0.57$), while including tank in the model. Overall, there were no significant effects of early-life conditioning on alpha or beta diversity of microbial communities in fish exposed to later life crowding stress.

3.6 Differential abundance analysis reveals taxon-specific microbial responses

The rainbow trout microbiota was dominated by two phyla, *Firmicutes* and *Proteobacteria*, which together accounted for approximately 95–98% of the total community composition (**Figure S12**). At the family level, *Lactobacillaceae* and *Enterobacteriaceae* were the most abundant taxa (Fig. 6).

To investigate the effects of early-life conditioning on the microbial communities under subsequent crowding stress, differential abundance analysis of ASVs was performed by comparing conditioned and unconditioned fish within the same later life stress treatment.

In diploid fish exposed to acute stress, differential abundance analysis identified four ASVs, comprising three ASVs belonging to the family *Lactobacillaceae* (phylum Firmicutes) one ASV from the family *Microbacteriaceae* (phylum *Actinobacteriota*) (**Table S1**). Under chronic stress, five differentially abundant ASVs were detected, including four ASVs from *Enterobacteriaceae* family (phylum *Proteobacteria*) and one ASV from *Lactobacillaceae* family (**Table S2**).

For triploid fish, no differentially abundant ASVs were identified between conditioned and unconditioned fish under acute stress, whereas chronic stress was associated with significant changes in the abundance of five ASVs belonging to the families *Enterobacteriaceae*, *Lactobacillaceae*, and *Streptococcaceae*.

3.7 Host–microbiota associations in conditioned fish exposed to later life crowding stress

To identify the potential host–microbiota interactions, we assessed the correlations between host gene expression and the abundance of bacterial ASVs. In diploid fish the analysis revealed two host genes *SLIT* and *NTRK-like family member 4* (*slitrk4*) and *synapsin-2* (*LOC110531996*) were negatively correlated with the abundance of seven *Lactobacillus* ASVs including *Lactobacillus secaliphilus* and *Lactobacillus amylovorus* (**Table S3**). These genes were involved in neuronal connectivity and synaptic transmission. In contrast, no significant host–microbiota interactions were observed in triploid fish.

4. Discussion

We investigated how early-life conditioning alters later life stress responses using rainbow trout (diploid and triploid) as study species exposed to common aquaculture husbandry-related stressors. We hypothesised that early-life conditioning by cold shock persistently alters transcriptional stress response. Further, the microbiome altered by early-life conditioning responds differentially to the later-life stress and may influence transcriptional response to stress. Our findings revealed that early-life conditioning significantly reduced the transcriptional related stress response to later life crowding stress compared to unconditioned fish and the responses were ploidy dependent. Conditioning had minimal lasting effects on the microbiome and did not influence subsequent microbiota response to later life stress.

Crowding stress induced significant transcriptional stress responses in unconditioned fish

Crowding stress induced significant changes in gene expression compared to non-stressed control fish. Transcriptional responses to crowding stress observed here were dependent on the duration of the exposure and the ploidy. For example, in diploid fish, we observed elevated immune related pathway activation, characterised by T-cell activation and adaptive immune responses (*cd3e, itk, il7r* and *eomesa*) in response to acute stress, whereas in chronic stress, the response was dominated by the oxidative phosphorylation, electron transport, ATP biosynthesis, cellular respiration, ribosome biogenesis, and protein folding pathways (*atp5f1d, atp5pf, ndufb3, ndufc2, ndufs4 and hspa14*). In triploids, the acute stress responses were related to adaptive immune responses, lipid and fatty acid transport (*apoa1, rbp2 and rbp4*), while under chronic stress triploid responses were related to enrichment of developmental signalling pathways (*ptch1* and *ptch2*). Together, these results indicate that crowding stress triggered cellular stress responses including immediate immune response to prolonged metabolic reprogramming in the unconditioned fish (Kültz. 2020; Somero. 2020). However, the nature of the response, underlying pathways and genes were different between diploid and triploid fish. The observed transcriptional stress responses in both ploidies are consistent with the previous findings in teleosts and show that crowding stress exposure causes activation of immune responses, elevated metabolic activity and influences signalling pathways (Santos et al. 2010; Ellison et al. 2020; He et al. 2021; Li et al. 2023).

Early-life conditioning attenuates the transcriptional stress response

Fish exposed to early-life conditioning had significantly reduced transcriptional stress response to later life stress compared to unconditioned fish. Similar reduction in transcriptional stress responses to later life stress following early-life mild stress has been documented in teleosts exposed to various stressors, including cold shock (Moghadam et al. 2017) and chemical stressors (Uren Webster et al. 2025). Along with the reduction in the total number of DEGs in conditioned fish, we observed that only 9.88–21.04% of genes were shared between the conditioned and unconditioned fish exposed to later life stress, indicating limited overlap in the molecular pathways during later life stress. Specifically, the proportions of shared genes in diploids was 9.88% for acute stress (1305/1448 unique genes) and 11.12% for chronic stress (879/989 unique genes), and for triploids 21.04% for acute stress (653/827 unique genes), and 6.85% for chronic stress (939/1008 unique genes). These findings indicate that early-life conditioning not only reduced the transcriptional activity, but also it reshaped the repertoire of genes involved. Such modulation of transcriptional response indicates that conditioning induces long term changes in stress coping mechanisms. In an earlier study (Moghadam et al. 2017), fish exposed to early-life cold shock during both pre- and post-hatching displayed more comparable transcriptomic profiles to control fish than fish which only received cold shock either pre or post hatching, indicating an adaptive transcriptional response to repetitive stress exposure during early developmental stages. Further, fish subjected to pre- and post-hatching not only reduced stress sensitivity but also enhanced the growth performance (Moghadam et al. 2017). This modulated transcriptional stress response has been linked to developmental plasticity mediated by epigenetic mechanisms, such as chromatin remodelling, DNA methylation and ncRNAs (Moghadam et al. 2015; Moghadam et al. 2017; Robinson et al. 2019; Liu et al. 2022b; Uren Webster et al. 2025).

Despite being exposed to the same later life stressors, following early life conditioning, diploid and triploid fish exhibited distinct transcriptional responses. Differences were observed not only in the magnitude of the transcriptional response but also in the identity of the genes responding to stress. Most ploidy comparative studies have reported differences in growth rate, transcriptome, methylome, stress and immune response (Beato et al. 2025; Keyvanshokooh. 2025; Mami et al. 2025), with triploid individuals being in general more sensitive to stress and showing reduced immune response (Madaro et al. 2024; Mahmoudi et al. 2025). Such differences are possibly due to fundamental biological differences linked with triploids, including changes in genome regulation and differences in cellular physiology (Wang et al. 2023; Keyvanshokooh. 2025). Although the specific genes and pathways involved may vary between diploid and triploid conditioned fish, reduction in magnitude of the transcriptional response shows that early-life conditioning may function as a regulator of transcriptional reprogramming, altering stress-coping mechanism independent of ploidy.

In unconditioned fish, crowding stress enriched pathways associated with ATP synthesis, cytoskeletal remodelling, and mitochondrial energy production; however, in conditioned fish, these responses were no longer enriched. This implies that when subjected to later life stress, conditioned fish need less substantial metabolic reprogramming. Similar findings have been reported in Atlantic salmon, where repeated early-life mild stress lowered cortisol response, changed immune gene expression, and balanced growth and stress responses to later-life stress (Moghadam et al. 2017; Robinson et al. 2019). Therefore, early-life conditioning may prime regulatory networks involved in maintaining physiological homeostasis, minimizing the energetic costs of responding to later-life stress (Moghadam et al. 2015; Moghadam et al. 2017; Robinson et al. 2019). Although these results support the possibility of an adaptive stress conditioning effect, further studies integrating physiological, phenotypic, and endocrine measurements are necessary to determine whether the attenuated transcriptional response in conditioned fish represents enhanced stress resilience and improved physiological performance.

Early-life conditioning does not drive microbiota structural changes to subsequent stressors

Overall, we observed differences in alpha diversity between ploidies, which was lower in triploid fish. In general, microbiota diversity is substantially affected by host factors (Cai & Wei. 2023; Small et al. 2023; Anka et al. 2025) as well as environmental factors (Sullam et al. 2012; Kim et al. 2021; Shankregowda et al. 2023). In *Carassius auratus*, for example, triploidisation can affect fish intestinal traits (structure, relative mass and length) along with the gut microbiota, which in turn can influence fitness-related traits, such as growth rate (Wang et al. 2023) and decrease species richness compared to diploid fish (Cai & Wei. 2023), in line with the results here. The observed differences in alpha diversity in triploids could be related to host factors, including immune response, stress response or intestinal morphological traits induced by the triploidisation, as experiments were conducted in controlled environment with minimal environmental influence (Cantas et al. 2011; Wang et al. 2023).

We had previously found that early-life conditioning significantly affected microbiota alpha diversity (Shannon and Simpson diversity) but not beta diversity (*Overland et al. under review*). As found in this study, acute and chronic stress effects seemed to have opposite directions, which masked their individual effects on the alpha diversity (except acute stress in Chao1 richness). In contrast, we detected a significant effect when considering the combined impact of crowding stress, suggesting that the cumulative effect of crowding stress may have a stronger influence on microbial diversity than individual stressors alone. In general, stress exposure is known to disrupt the microbiota stability and composition, (Zhou et al. 2022; Fang et al. 2023; Liu et al. 2023; Kuizenga et al. 2025) leading to dysbiosis (Fang et al. 2023), which in turn adversely affects the host health and immune function (Lin et al. 2020). Conversely, a diverse and healthy microbiota contributes to host fitness by promoting immune function (Gómez & Balcázar. 2008), maintaining physiological homeostasis, and improving resistance to pathogens (Bozzi et al. 2021) and environmental stressors (Houwenhuyse et al. 2021).

Several studies have reported that early-life stressors can induce persistent microbiome changes through priority effects (Sprockett et al. 2018; Debray et al. 2022; Román et al. 2025), whereby initial colonization influences the subsequent assembly and stability of microbial communities (Debray et al. 2022). However, contrary to our expectations, the interaction between early-life conditioning and later-life crowding stress was not significant, suggesting that the microbiome response to subsequent stress may not simply reflect the additive effects of successive stressors. One possible explanation is that early-life conditioning altered the developmental trajectory of the microbiota, resulting in a community that was either more resilient or less responsive to later environmental perturbations. Together, these findings highlight the sensitivity and plasticity of the microbiome during early developmental stages demonstrating that early-life conditioning might play a vital role in shaping long-term host–microbiota interactions in response to environmental stressors (Alberdi et al. 2016; Houwenhuyse et al. 2021; Ratsika et al. 2021).

Conclusions

Our study revealed that early-life environmental conditions can significantly modulate later life stress responses. Early-life conditioning by cold shock significantly reduced later life transcriptional stress responses, indicating that early-life experiences can reprogram host stress responsiveness. Despite ploidy-specific differences in transcriptional responses to crowding stress, early-life conditioning reduced the transcriptional stress response in both diploid and triploid fish, suggesting that developmental programming similarly modulates later-life stress responses. While early-life conditioning and crowding stress independently altered microbial community structure, early-life conditioning did not significantly modify the microbiome response to subsequent crowding stress, suggesting that microbial responses to later life stressors are more complex than simple additive effects. Together, these findings suggest that early-life experiences can have persistent effects on host physiology while influencing the microbiome and host transcriptome through distinct mechanisms.

Authors' contributions

AMS – Conceptualization, Methodology, Formal analysis, Visualization, Validation, and Writing - original draft

BO – Experimental design, Methodology, Validation, Writing - review & editing

ALH – Sequencing, Writing - review & editing

MH – Sequencing, Writing - review & editing

CA – Sequencing and Resources

RS – Sequencing and Resources

CGL – Experimental design, Conceptualization, Validation, Writing - review & editing

TMW – Experimental design, Conceptualization, Validation, Supervision, Resources, Writing - review & editing

SC – Experimental design, Conceptualization, Validation, Funding acquisition, Supervision, Resources, Project administration, Writing - review & editing

Declarations

Conflict of interest declaration

The authors declare that there are no conflicts of interest.

Ethics declarations

Ethics, Consent to Participate, and Consent to Publish declarations: not applicable.

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Author Contribution

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Data Availability

The data presented in the study are deposited in the Sequence Read Archive (SRA) repository, accession number PRJNA1503184.

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Figures

Figure 1

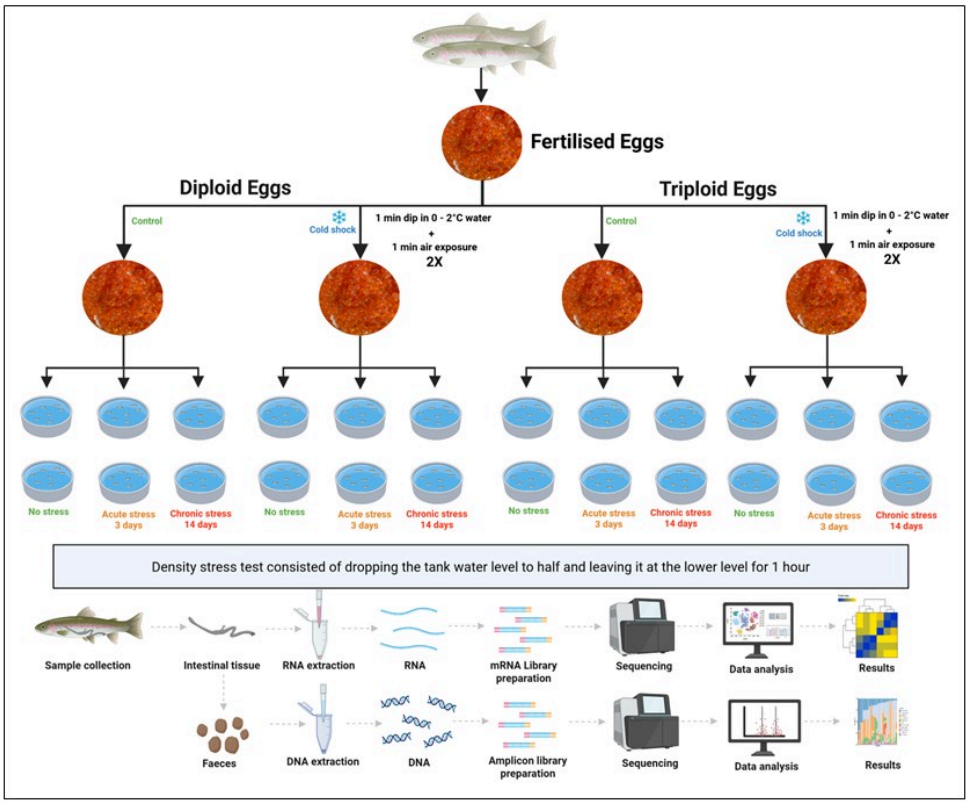


Figure 1

Schematic representation of the experimental design for diploid and triploid rainbow trout under early-life conditioning (cold shock) and subsequent crowding stress exposure. Downstream workflows included sample collection, DNA and RNA extraction, library preparation, sequencing and bioinformatics analysis.

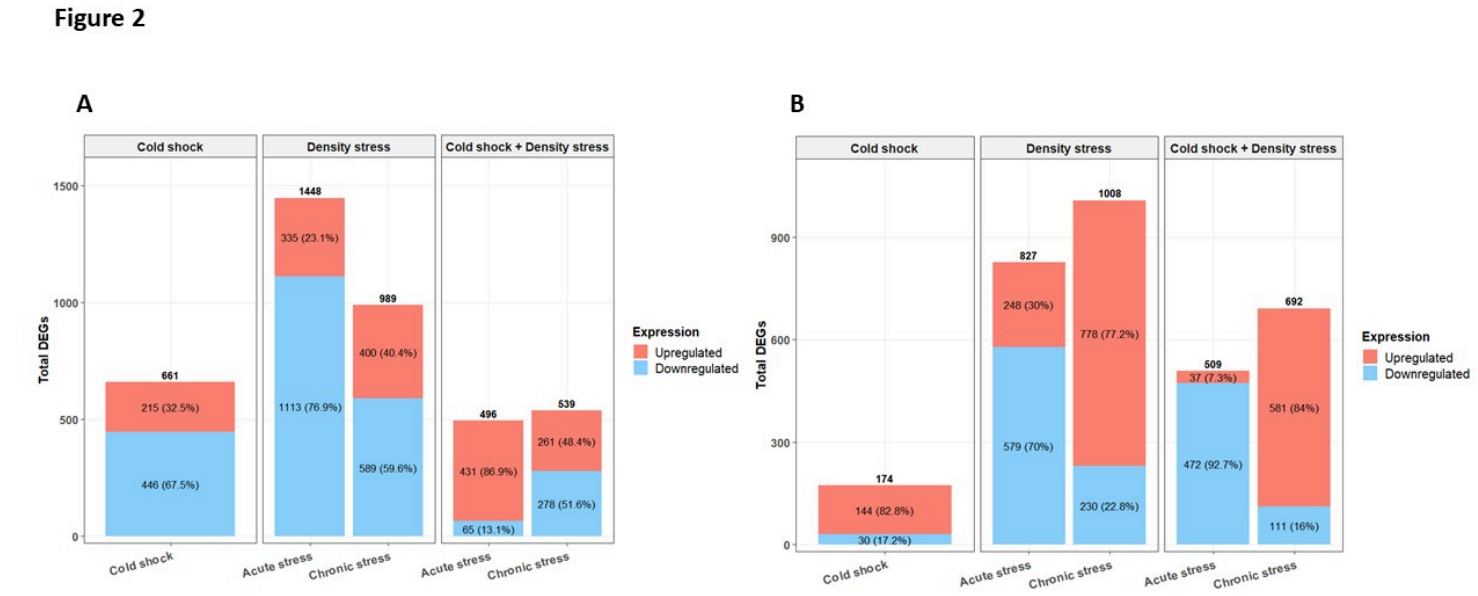


Figure 2

Differential gene expression profiles in diploid and triploid fish under different treatment conditions. Number of up- and downregulated DEGs in diploid (A) and triploid (B) conditioned (cold shock) and unconditioned fish exposed to acute and chronic crowding stress.

Figure 3

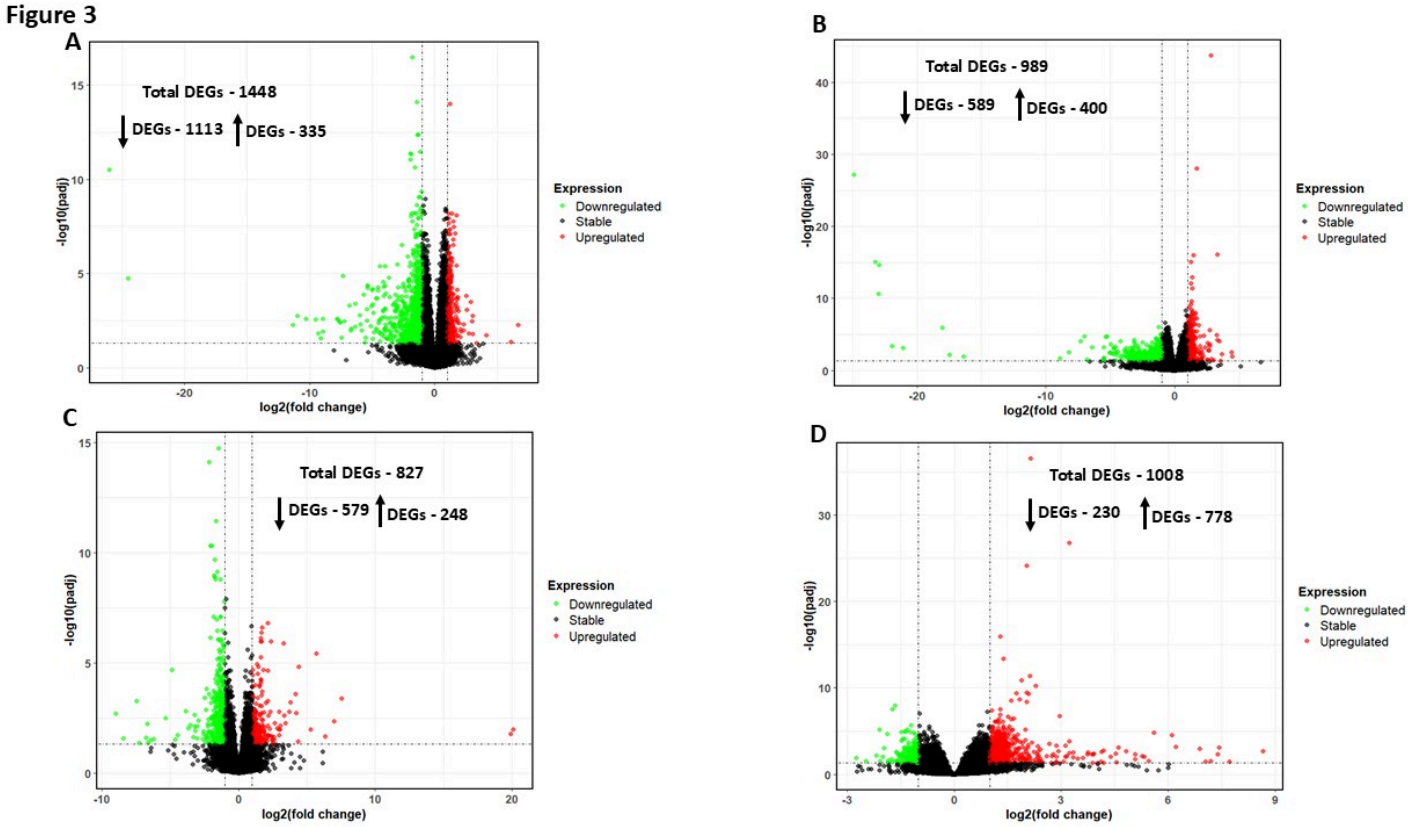


Figure 3

Differential gene expression analysis of diploid and triploid unconditioned fish exposed to crowding stress. (A) Volcano plot illustrating the differentially expressed upregulated (red) and downregulated (green) genes in diploid acute crowding stressed (a), diploid chronic crowding stressed (b), triploid acute crowding stressed (c) and triploid chronic crowding stressed (d) compared with control fish. Genes were considered significantly differentially expressed at |Log2 fold-change| ≥ 1 and p value < 0.05.

Figure 4

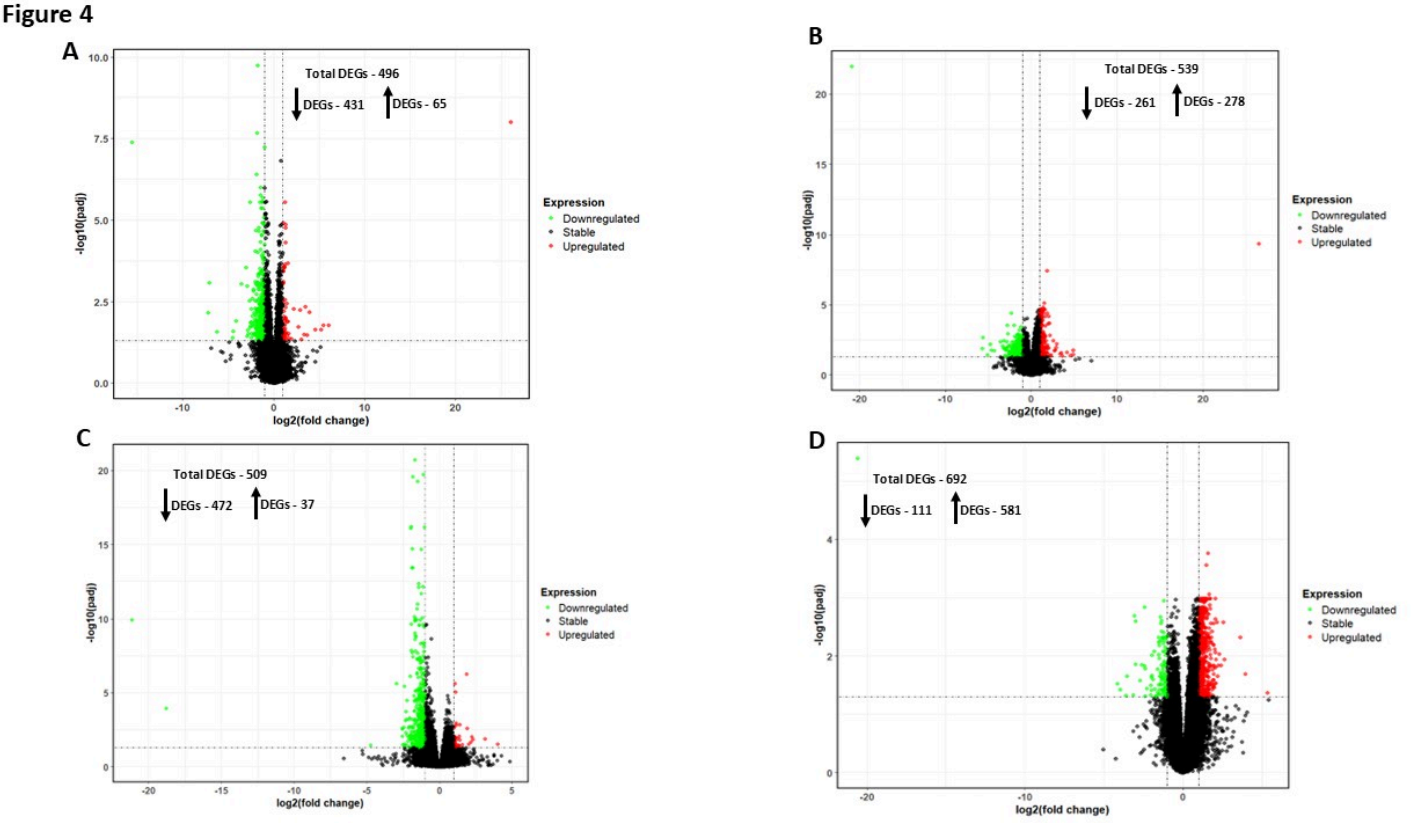


Figure 4

Differential gene expression analysis of diploid and triploid conditioned fish exposed to crowding stress. (A) Volcano plot illustrating the differentially expressed upregulated (red) and downregulated (green) genes in diploid conditioned fish acute crowding stressed (a), diploid conditioned fish chronic crowding stressed (b), triploid conditioned fish acute crowding stressed (c) and triploid conditioned fish chronic crowding stressed (d) compared with control fish. Genes were considered significantly differentially expressed at |Log2 fold-change| ≥ 1 and p value < 0.05.

Figure 5

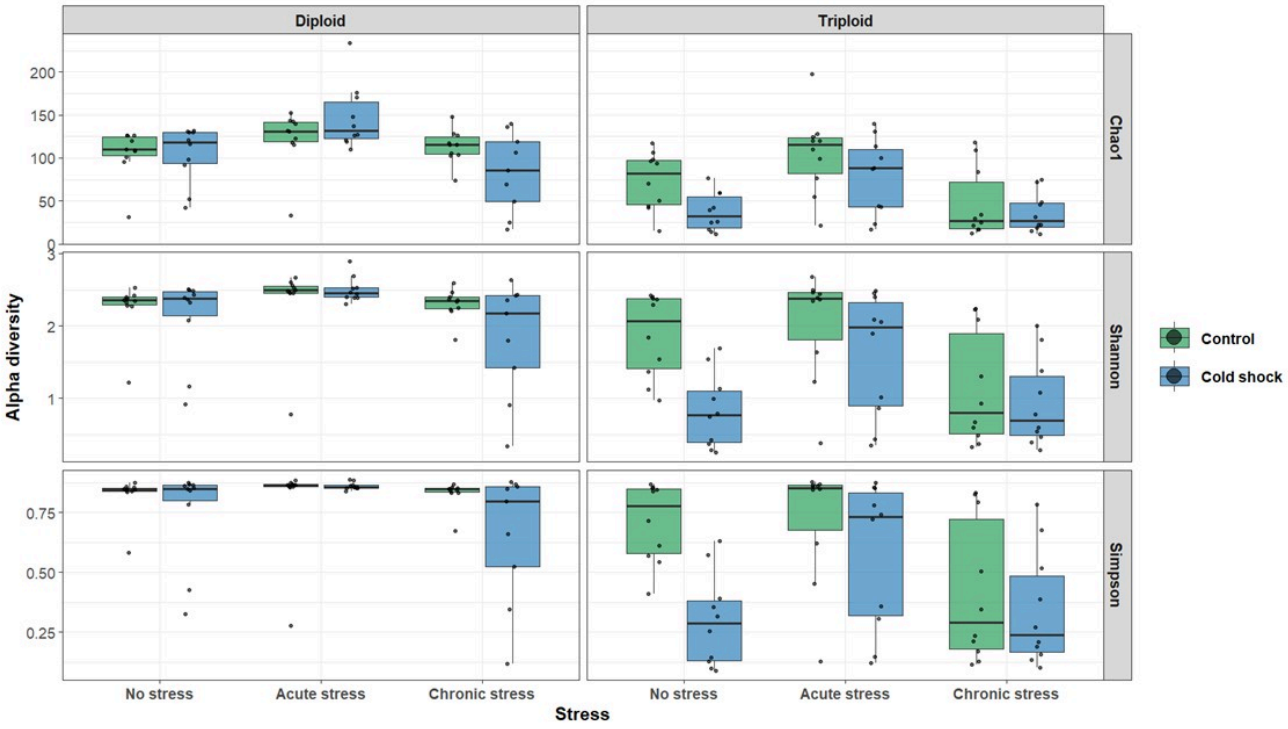


Figure 5

Alpha diversity metrics (Chao1 richness, Shannon diversity, and Simpson diversity) of the gut microbiota in diploid and triploid rainbow trout exposed to early-life conditioning (cold shock) and later-life crowding stress (acute and chronic stress).

Figure 6

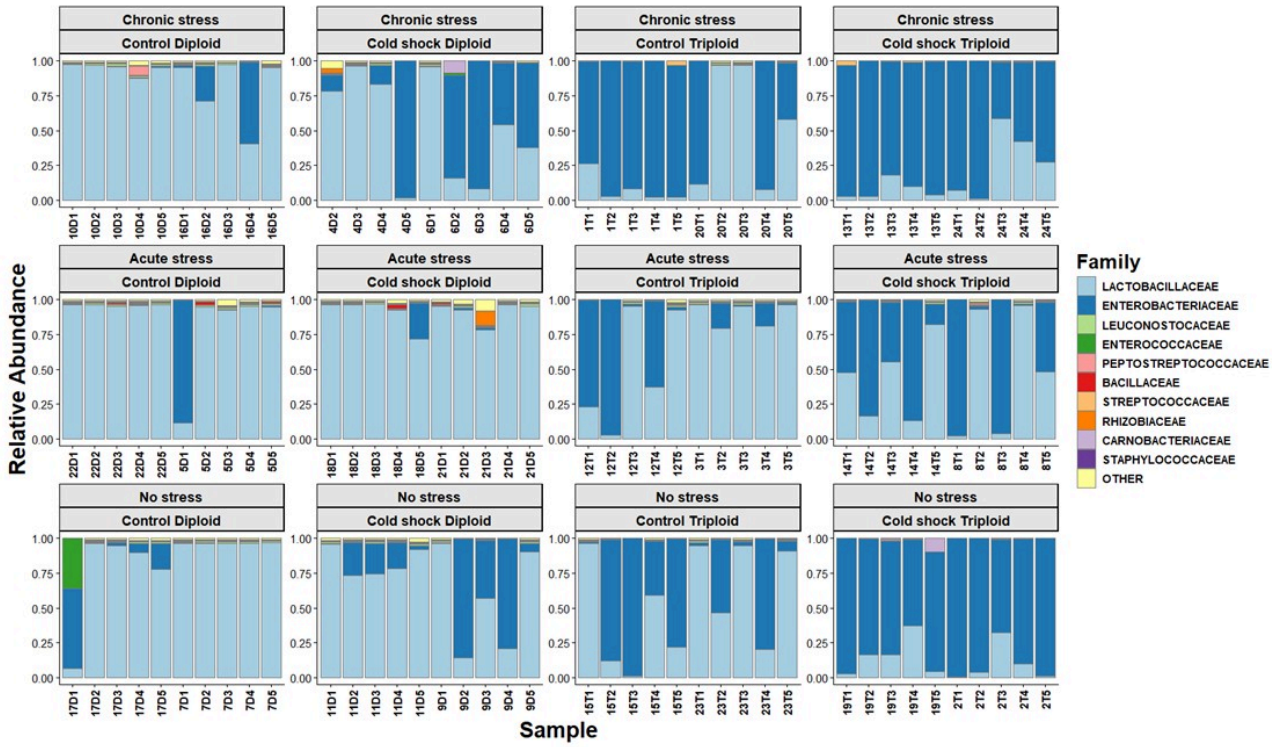


Figure 6

Relative abundance of dominant bacterial family in the gut microbiota of diploid and triploid rainbow trout across crowding stress treatments and early-life conditioning.

Supplementary Files

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