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Coding/non-coding cross-talk in intestinal epithelium transcriptome gives insights on how fish respond to stocking density☆

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A B S T R A C T

Fish respond to increasing stocking density as a stressor, adjusting physiological functions to increase energy supply for coping with deleterious effects and adapting. These responses are complex and systemic, and the molecular mechanisms involved remain undetermined. One of the most sensitive organs to environmental and homeostatic disruptions is the intestine, and since it plays several vital functions, understanding the molecular underpinnings of this organ under deleterious conditions is imperative for health improvement in aquaculture systems. This study aimed to understand how different stocking densities of rainbow trout (*Oncorhynchus mykiss*) modulates the coding and non-coding RNAs profiling, that in turn, play key roles to maintain the fish homeostasis. For this, the intestine tissue of juvenile trout stocked for 30 days either at low (LD: 3 kg m^{-3}) or high density (HD: 40 kg m^{-3}) were sampled to isolate total RNA and then construct cDNA libraries for an illumina sequencing platform. Differential gene expression analysis revealed a generalized downregulation of transcripts, including coding and long non-coding RNAs (lncRNAs). Notably, significant differences in transcripts involved in metabolic pathways, as well as immune and epithelium integrity and stability related pathways were found. A high number of downregulated transcripts enriched these pathways, and a strong correlation was observed between the most differentially expressed transcripts and the highly expressed lncRNAs. This study suggests a cross-talk between coding and non-coding RNAs in the intestine of fish exposed to suboptimal conditions, providing new insights into the regulatory role of the lncRNAs on fish response to stressors.

1. Introduction

Farmed fish are permanently exposed to suboptimal artificial culture conditions that could lead to deleterious effects by stress (Ellis et al., 2002; Kumar et al., 2015). Stress responses in fish have long been addressed as a complex network of biological alterations with a high degree of conservation with higher vertebrates (Bonga, 1997; Schreck and Tort, 2016). Although theoretically all tissues might be targeted, liver, gills, and intestine are primarily affected (Bonga, 1997; Faught et al., 2016; Schreck and Tort, 2016). There is particular interest in the intestine because it contains a complex and dynamic microbiota community and it plays an active role on the modulation of neuronal responses as being a member of the gut-brain axis (Jonsson and Holmgren, 2011; Gomez et al., 2013; Nardocci et al., 2014; Salinas and Parra, 2015; Rosengren et al., 2017). Also, fish intestines have been shown to exhibit molecular responses associated to stress through activation of glucocorticoid receptors, suggesting that cortisol might have a direct effect on the tissue (Takahashi et al., 2006). Although there is still little information on the direct effects of stress hormones on the intestine, it is known that environmental stress severely affects intestine stability and homeostasis, jeopardizing its functions (Sundh et al., 2010; Sundh and Sundell, 2015; Rosengren et al., 2017).

Nevertheless, how intestines respond to deleterious conditions is still poorly understood, especially when it comes to the molecular response to a stressor. For instance, Xia et al. (2013) showed that stress-responsive intestine transcriptome of the Asian seabass (*Lates calcarifer*) after a challenge with LPS, *Vibrio harveyi*, salinity, and fasting, involves the modulation of over 200 pathways for each stressor, and 37 genes highly regulated and shared between multiple stressors. This study highlighted the complex coordination occurring in stress-responsive tissues. Similarly, we reported the complexity of the immune-related transcriptomic response in rainbow trout (*Oncorhynchus mykiss*) intestine under high stocking density, and how functional diets could modulate this response mitigating density effects on immunity (Gonçalves et al., 2017). Although this last study pointed out the complexity of intestine transcriptomic regulation, molecular mechanisms regulating these responses remains uncovered in fish.

It has been proposed that stress response and intestinal integrity and functioning be regulated by epigenetic processes such as DNA methylation and non-coding RNAs post-transcriptional regulation (Leung and Sharp, 2010; Runtsch et al., 2014; Xiao et al., 2016; Wang et al., 2017). Among ncRNAs, long non-coding RNAs (lncRNAs) are the most studied (Mattick and Rinn, 2015) and have been shown to be involved in several processes in fish such as the response to pathogenic bacteria

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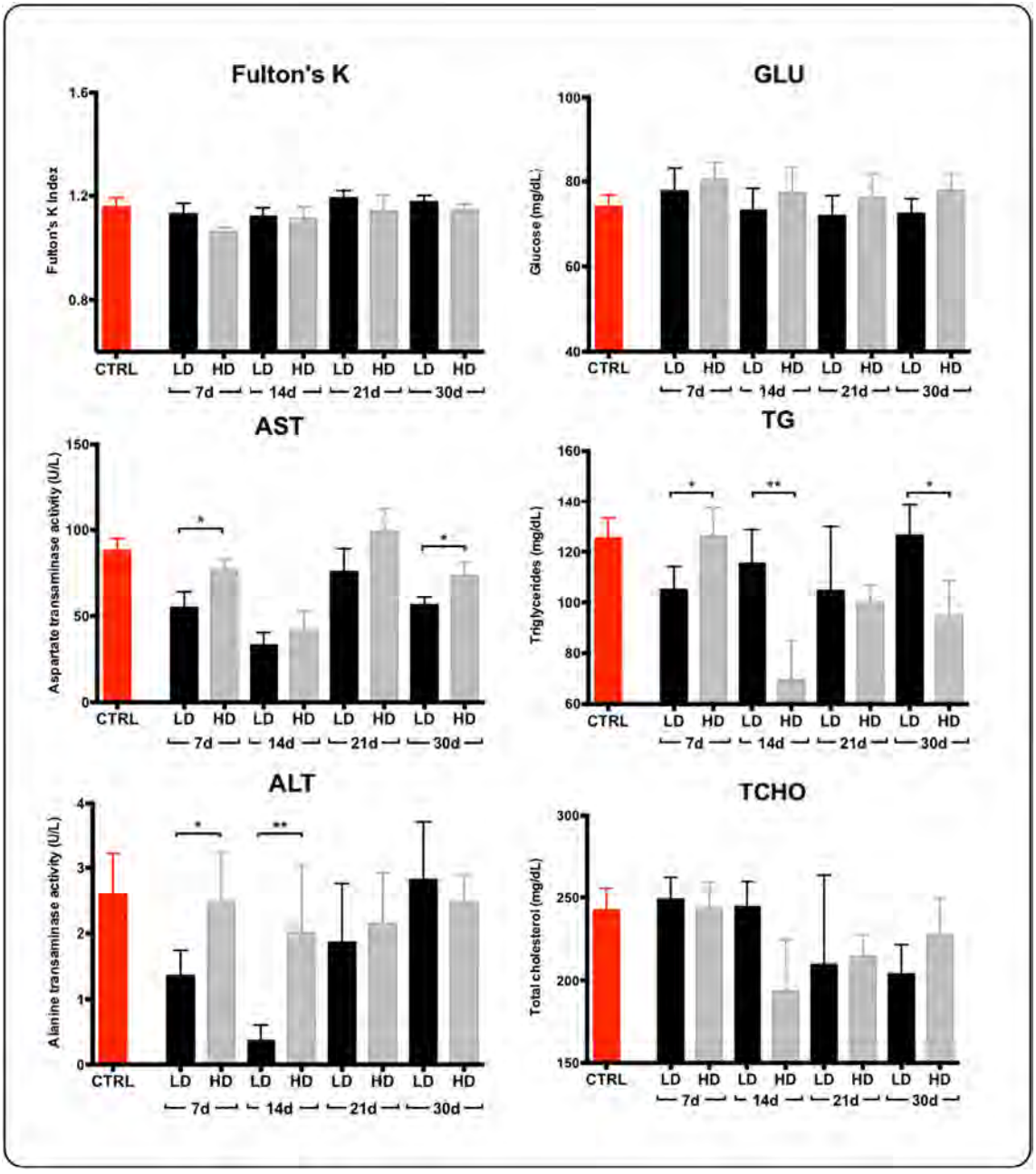


Fig. 1. Physiological output of juvenile rainbow trout (*Oncorhynchus mykiss*) stocked for 30 days at low (LD = 3 kg m^{-3}) and high density (HD = 40 kg m^{-3}). Bars indicate mean ± SE and asterisks indicate significant differences between LD and HD at the time (2-way ANOVA; Tukey HSD; n = 5–7/replica).

(Valenzuela-Miranda and Gallardo-Escarate, 2016; Tarifeño-Saldivia et al., 2017; Valenzuela-Miranda et al., 2017), virus (Boltaña et al., 2016) and ectoparasitic infections (Valenzuela-Muñoz et al., 2017). For instance, Nuñez-Acuña et al. (2017) reported a strong modulation of intestinal lncRNAs in response to functional diets in rainbow trout (*Oncorhynchus mykiss*), with a strong correlation between expression of lncRNAs and mRNAs associated to metabolism, immune response and other functions commonly modulated by functional supplements. However, the implication of lncRNAs in the regulation of intestinal response to deleterious conditions is yet to be studied. This study aimed to evaluate the differential intestinal transcriptomic response of rainbow trout kept under different stocking densities as a proxy for chronic stress-response, using RNA-seq analysis. The study also aimed to perform the first screening of non-coding RNAs expression related to chronic stress and potential regulation of coding RNAs, identifying the most key pathways involved in the response.

2. Material and methods

2.1. Samples and culture conditions

Freshwater-cultured juvenile rainbow trout (*O. mykiss*, N = 160) with an average weight of 34.3 g (± 5.8 g) were obtained from a commercial fish farm (Huililco, Chile) located in the Araucanía Region of Chile. Fish were screened for health conditions and certified free of the most prevalent pathogens. Healthy fish were randomly transferred to a recirculating system (1800 L of total volume) with four circular tanks (325 L). Fish were acclimated for 14 days to a natural photoperiod to maintain similar conditions as in origin, and water parameters were kept optimal with 10 ± 0.5 °C, pH 7.5, ammonium levels below detection limits. These conditions were kept throughout the experiment. Fish were fed once daily with a commercial diet micro50 (Ewos-Cargill®, Coronel, Chile) in a proportion of 1.3% total biomass for each

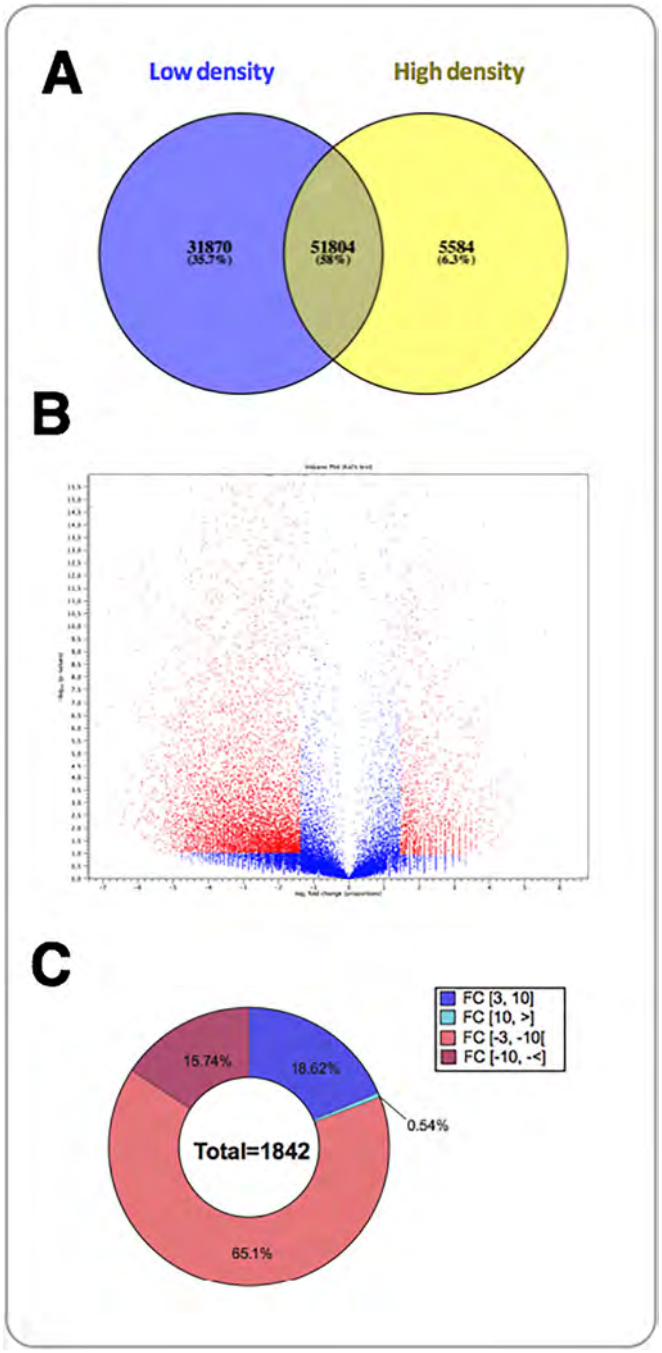


Fig. 2. Comparison of the *O. mykiss* intestinal transcriptomic response to high and low density. A. Venn diagram representing the number of contigs, a total gene read > 10, that are shared or unique to each condition (LD: low density, HD: high density). B. Volcano plot, identifying in red the coding genes selected and considered differentially expressed between both conditions (FDR corrected p-value < 0.05 and FC ≥|3|; Kal's proportion test). C. Pie chart representing the number of genes up and down-regulated (total gene read > 10, FDR corrected p-value < 0.05 and FC ≥|3|) in HD compared to LD.

tank, which proximate compositions were 48% of crude protein, 16% lipids, 11% humidity, 11% ash and 2.5% crude fiber. All animals used in this study were treated following the Biosecurity Regulations and Ethical Protocols approved by the University of Concepcion Ethics Committee as a mandatory part of the FONDECYT program granted by the CONICYT-Chile.

2.2. Experimental design

To simulate density conditions without loss of water quality, we placed a plastic net in two tanks in the recirculating system. By doing this, the height of the water column was reduced, keeping fish at high stocking density (HD: 40 kg m^{-3}), whereas density in other two tanks was kept low (LD: 3 kg m^{-3}). To evaluate health condition along experimental period (at 7, 14 and 21 days), fish (n = 5) were randomly collected from each tank, anesthetized with clove oil (0.3 mL 10% eugenol/L), weighed, and sized to calculate Fulton's condition factor (K) following the equation K = (Total weight (g) / Fork length (mm) $* 10^3$). Then, blood samples were taken from the caudal vein using a syringe treated with an anticoagulant (0.1% EDTA) and were centrifuged (10 min, 1500 × *g*) to collect plasma, which was stored at −20 °C until clinical biochemistry analysis. Sampling net size was adjusted to account for the biomass reduction, and the tissues sampled as above described. At the end of the experiment, 30 days after the start of the trial, ten fish per tank were sampled. In addition to blood collection, the whole intestines were carefully dissected. Then, a section of 1 cm of anterior and posterior intestine were cleaned from fecal contents suspended in RNA Later (Ambion®, Thermo Fisher Scientific, Waltham, MA, USA), and stored at −80 °C.

2.3. Plasma biochemical parameters

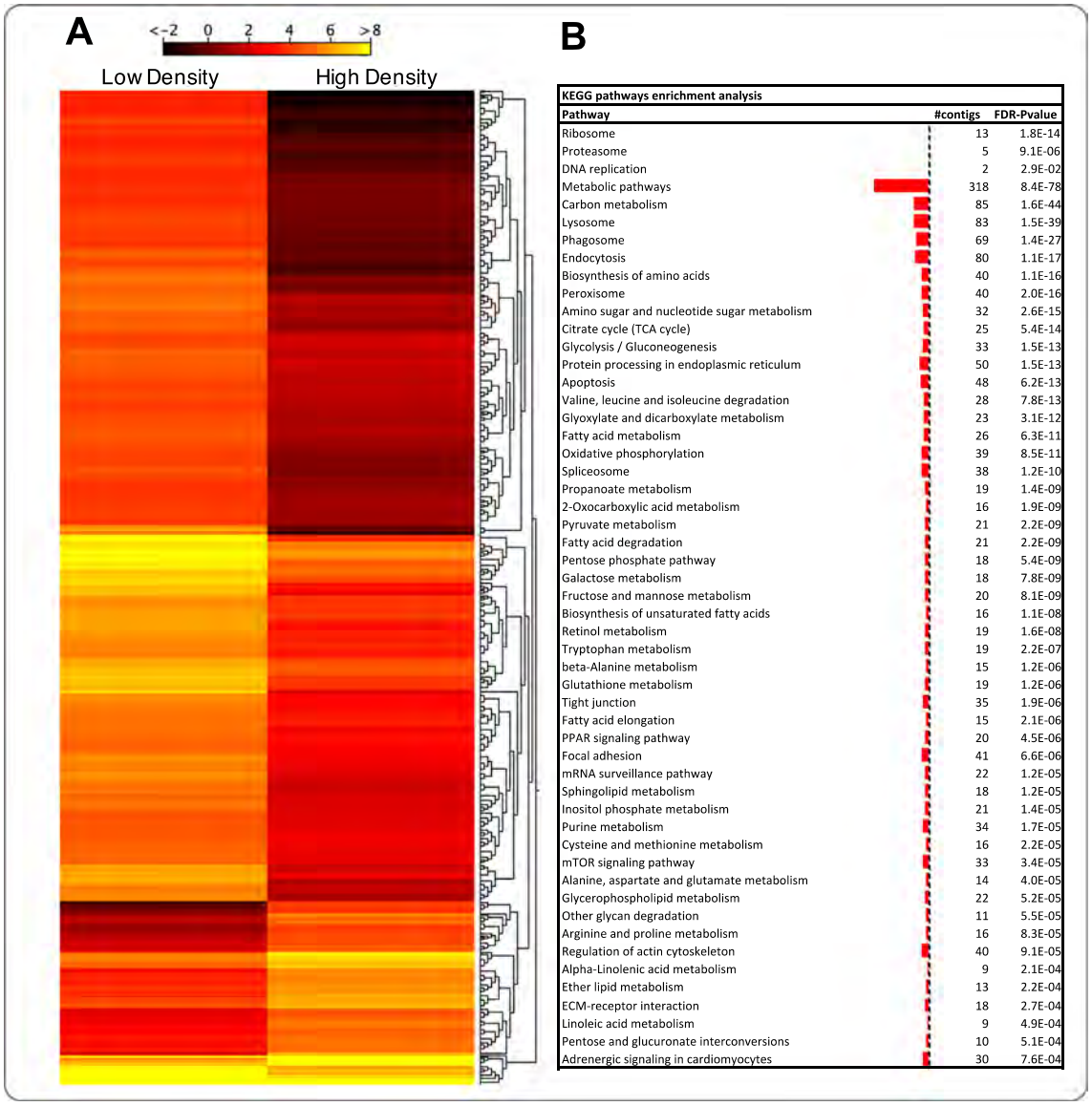
The collected plasma samples were used to assess plasma concentrations for the following: glucose (HUMAN Diagnostics Kit; Wiesbaden, Germany), triglycerides, total cholesterol, alanine and aspartate transaminase (Labtest Diagnosticos; Labtest, Brazil). All protocols were carried out according to the manufacturer's instructions, using a microplate spectrophotometer Multiscan GO (Thermo, Waltham, USA).

2.4. RNA extraction and sequencing

Total RNA from both intestine sections (anterior and posterior) was extracted using Trizol Reagent® (InvitrogenTM, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Concentration and purity of total RNA were analyzed in a Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies, USA), and quality of isolated RNA was measured in a TapeStation 2200 using the R6K Reagent Kit according to the manufacturer's instructions (Agilent Technologies Inc., USA). Samples with high purity and with a RIN over 8.0 were selected for subsequent analysis. Construction of cDNA libraries for each experimental group (HD or LD) was performed using the TruSeq RNA Sample Preparation Kit v2 (Illumina, USA) according to fabricant instructions. Samples that passed the quality filter and had RIN > 8 were selected (n = 6 per group) from each HD and LD groups. Anterior and posterior intestine regions were pooled in two biological replicates and separately sequenced by the MiSeq (Illumina, USA) platform using MiSeq v2 Reagent Kit with paired-end runs of 500 cycles at the dependencies of the Laboratory of Biotechnology and Aquatic Genomics, Interdisciplinary Center for Aquaculture Research (INCAR), University of Concepción, Chile. Data generated from RNA sequencing were processed using the CLC Genomic Workbench software (version 10.0, CLC Bio, Denmark). Raw data from each library were trimmed for adapters and filtered by the quality and read sequences were de novo assembled in a single file containing the two replicates of the two experimental groups. De novo assembly was performed as described by Gonçalves et al. (2017) using a mismatch cost of 2, insert cost of 3, a minimum contig length of 200 bp, a similarity of 0.8 and a trimming quality score of 0.05.

2.5. Transcriptome analysis – coding RNAs

RNA-seq analysis was performed to compare the intestinal



KEGG pathways enrichment analysis		
Pathway	#contigs	FDR-Pvalue
Ribosome	13	1.8E-14
Proteasome	5	9.1E-06
DNA replication	2	2.9E-02
Metabolic pathways	318	8.4E-78
Carbon metabolism	85	1.6E-44
Lysosome	83	1.5E-39
Phagosome	69	1.4E-27
Endocytosis	80	1.1E-17
Biosynthesis of amino acids	40	1.1E-16
Peroxisome	40	2.0E-16
Amino sugar and nucleotide sugar metabolism	32	2.6E-15
Citrate cycle (TCA cycle)	25	5.4E-14
Glycolysis / Gluconeogenesis	33	1.5E-13
Protein processing in endoplasmic reticulum	50	1.5E-13
Apoptosis	48	6.2E-13
Valine, leucine and isoleucine degradation	28	7.8E-13
Glyoxylate and dicarboxylate metabolism	23	3.1E-12
Fatty acid metabolism	26	6.3E-11
Oxidative phosphorylation	39	8.5E-11
Spliceosome	38	1.2E-10
Propanoate metabolism	19	1.4E-09
2-Oxocarboxylic acid metabolism	16	1.9E-09
Pyruvate metabolism	21	2.2E-09
Fatty acid degradation	21	2.2E-09
Pentose phosphate pathway	18	5.4E-09
Galactose metabolism	18	7.8E-09
Fructose and mannose metabolism	20	8.1E-09
Biosynthesis of unsaturated fatty acids	16	1.1E-08
Retinol metabolism	19	1.6E-08
Tryptophan metabolism	19	2.2E-07
beta-Alanine metabolism	15	1.2E-06
Glutathione metabolism	19	1.2E-06
Tight junction	35	1.9E-06
Fatty acid elongation	15	2.1E-06
PPAR signaling pathway	20	4.5E-06
Focal adhesion	41	6.6E-06
mRNA surveillance pathway	22	1.2E-05
Sphingolipid metabolism	18	1.2E-05
Inositol phosphate metabolism	21	1.4E-05
Purine metabolism	34	1.7E-05
Cysteine and methionine metabolism	16	2.2E-05
mTOR signaling pathway	33	3.4E-05
Alanine, aspartate and glutamate metabolism	14	4.0E-05
Glycerophospholipid metabolism	22	5.2E-05
Other glycan degradation	11	5.5E-05
Arginine and proline metabolism	16	8.3E-05
Regulation of actin cytoskeleton	40	9.1E-05
Alpha-Linolenic acid metabolism	9	2.1E-04
Ether lipid metabolism	13	2.2E-04
ECM-receptor interaction	18	2.7E-04
Linoleic acid metabolism	9	4.9E-04
Pentose and glucuronate interconversions	10	5.1E-04
Adrenergic signaling in cardiomyocytes	30	7.6E-04

Fig. 3. Heat-map (A) of significant differentially expressed transcripts in *O. mykiss* intestine when exposed to different densities (LD: low density, HD: high density; FDR corrected p-value < 0.05 and FC $\geq |3|$; Kal's test), and list of the top 50 enriched pathways according to KEGG, the red bar represents down-regulated processes while green bars show up-regulated processes (B).

transcriptome of fish kept at low or high density, highlighting the differentially expressed genes. For this, the raw trimmed data of each dataset (experimental group replicates) were mapped against the consensus sequences from assembled contigs as a reference. RNA-seq settings were a minimum length fraction of 0.8 and a minimum similarity fraction of 0.9. The expression unit was set as transcripts per million of mapped reads (TPM) and was calculated in the two transcriptomes. Proportion based statistical analysis was applied to compare the groups and Kal's proportions statistical test was applied to TPM values of each transcriptome to evaluate fold change and distinguish differentially expressed transcripts. As initial screening, a volcano plot was performed to filter all the differentially expressed transcripts with FC $\geq |3|$, and FDR corrected p value < 0.05. All contigs that presented less than ten total gene reads in both conditions were eliminated from subsequent analysis to avoid misinterpretation of possible sequencing errors. Contigs passing these filters were clustered by hierarchical clustering of expression values, through calculation of Manhattan's distances and using average linkage, and presented in a heat map. The clustered contigs were annotated by BLASTx with the bony fish protein database available at UniProt (http://www.uniprot.org) and were analyzed for KEGG pathways and GO terms enrichment using KOBAS 3.0 (http://kobas.cbi.pku.edu.cn) with a cut-off of corrected p-value < 0.05. Selected pathways from the immunity system, epithelial integrity and metabolism were further evaluated in detail for expression patterns among the related genes.

2.6. Transcriptome analysis –non-coding RNAs

The expression level of previously identified lncRNAs (see Nuñez-Acuña et al. (2017)) was calculated in silico in the rainbow trout *O. mykiss* in high or low density using the CLC Genomics Workbench software (v.10.0 CLC Bio). Briefly, transcripts per millions of mapped reads (TPM) were calculated in the two transcriptome datasets (high and low density, HD and LD respectively). Parameters used for this analysis were as follows: match score = 1, mismatch cost = 2, insertion cost = 3, deletion cost = 3, length fraction = 0.5, and similarity fraction = 0.8. Then, a Kal's statistical test was applied to TPM values, to obtain the fold change values of the HD treatment vs. the LD treatment. LncRNA expression was considered significant when fold changes between LD and HD were $> |3|$ and when FDR-corrected p-values $<$

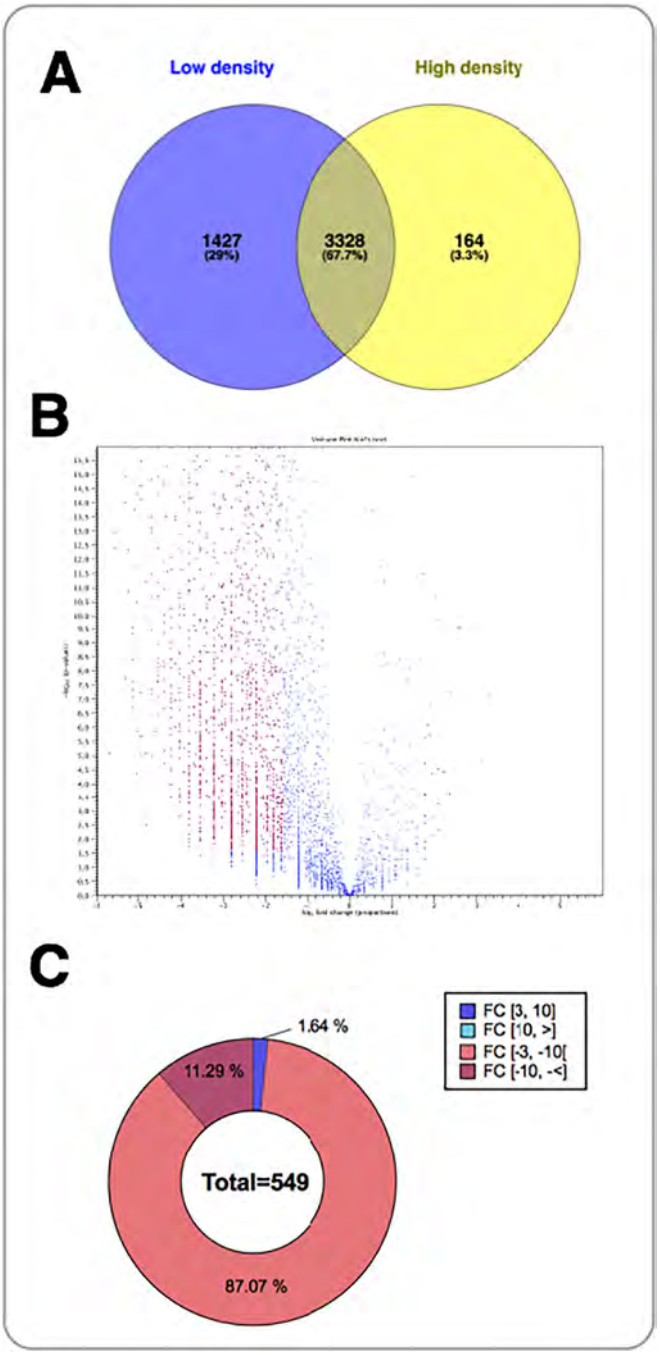


Fig. 4. Comparative expression of the *O. mykiss* intestinal lncRNAs response. A. Venn diagram representing the number of lncRNAs that are shared or unique to each condition. B. Volcano plot, with, in red the lncRNAs selected and considered differentially expressed between both conditions (FDR corrected p-value < 0.05 and FC $\geq |3|$; Kal's proportion test) C. Pie chart representing the number of differentially expressed lncRNAs up and down-regulated (total gene read > 10, FDR corrected p-value < 0.05 and FC $\geq |3|$) in HD compared to LD.

0.05. Fold change expression values were visualized through a volcano-plot. For further comparisons between treatment, a Venn diagram was made to obtain the number of significantly expressed lncRNAs, which were shared among groups or unique by treatment.

2.7. Data statistical analysis

Physiological data were screened for outliers, and parametric standards were evaluated with the Shapiro-Wilk's test (for normality) and with Levene's test (for variance homogeneity), and all variables were normalized with Box-Cox transformation using the JMP statistical software (v11.0; SAS Institute, NC, USA). A 2-way ANOVA was performed to infer differences among groups using time and density as fixed factors. When differences were significant, multiple comparisons post hoc Tukey's HSD test was performed do further depict. Differentially expressed coding and noncoding RNAs were identified using Kal's proportion Z test (Kal et al., 1999) included in CLC Genomics Workbench software (v10.0, CLC Bio, Aarhus, Denmark). To perform a correlation between most differentially expressed coding and non-coding RNAs we selected coding sequences according to their involvement in the following biological processes: Epithelial integrity, immunity and metabolism and lncRNAs that exhibit an FC $> |5|$ and FDR-corrected p-values < 0.05. Then, a Pearson's correlation was calculated from the TPM values of the selected coding genes and lncRNAs using the Corrplot package in the R v0.77 software (http://www.R-project.org), following Nuñez-Acuña et al. (2017).

3. Results

3.1. Physiological output

No mortality was observed throughout the experiment, and condition factor and plasma biochemical indicators (Fig. 1). No differences were observed on Fulton's K index along the experiment, and this ranged between 0.95 and 1.32 averaging 1.14 ± 0.04. High density increased plasma aspartate (AST) and alanine transaminase (ALT) activity, with a significant increase of AST after 7 and 30 days (density as significant factor $p < 0.01$), whereas ALT increase was significant at 7 and 14 days. Density effect was not significant in plasma glucose concentration, but an overall increasing pattern was observed in fish stocked at high density (density as significant factor $p = 0.048$). After seven days, fish stocked at high density presented higher plasma triglycerides concentration, followed by a significant decrease at 14 days, remaining lower than in LD group at 30 days, whereas total cholesterol levels did not differ among the two groups.

3.2. Intestinal transcriptomic response - coding RNAs

Illumina MiSeq sequencing of intestines of fish kept at different densities resulted in > 32 million reads (Table 1), and contigs from each group presented average 608 and 488 bp in LD and HD conditions respectively. From all the transcripts that were expressed in intestines of fish from LD and HD groups, 58% were shared (Fig. 2A). Interestingly, fish at low stocking density presented 31,870 transcripts (35.7%) that were not found in fish at high density, which presented only 6.3% exclusive transcripts. From all the differentially expressed contigs in both groups, a high number in the HD group are downregulated (Fig. 2B). From those, 65% expressed 3 to 10-fold less, and 15.7% were downregulated > 10-fold compared to fish at LD (Fig. 2C). This general downregulation was evidenced by hierarchical clustering of the differentially expressed genes with a strong highlighted cluster (upper cluster in heat map Fig. 3A). KEGG pathway enrichment analysis revealed that from all significantly enriched pathways, 96% were enriched by downregulated transcripts in HD group (Fig. 3B) (80 pathways with significant enrichment Table 1S). Among these, it was evident the enrichment of metabolic pathways such as glycolysis/gluconeogenesis, fatty acid metabolism, TCA cycle, biosynthesis and metabolism of amino acids, but also some defense-related pathways such as lysosome, phagosome, toll-like receptors, as well as pathways related to epithelial integrity and functioning such as tight junction, focal adhesion, ECM receptor interaction, gap and adherents junction (see Table 1S for complete list). GO enrichment analysis was concomitant with these results showing 570 terms enriched with downregulated transcripts in HD, more than five-fold the terms enriched with

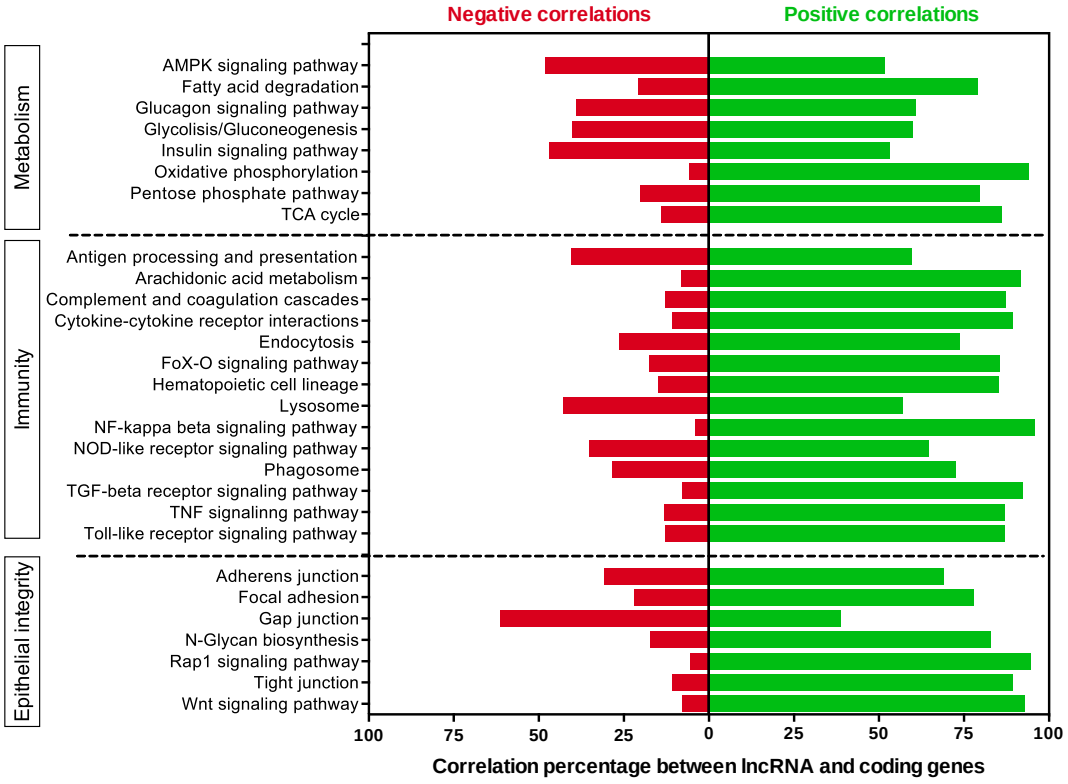


Fig. 5. Percentage of correlation between highly expressed coding genes and lncRNAs involved in three main processes: Metabolism, immunity and epithelial integrity.

upregulated transcripts (Table 2S). Several cell processes, metabolic processes (see Fig. 1S for global enrichment comparison), transport, membrane processes, defense-related processes, biological regulation, response to different stimulus among several others (Table 2S) were found to be significantly enriched with downregulated transcripts. This downregulation was also evident when highlighting the ten most regulated genes in metabolic response, immune response and epithelium integrity (Table 2). In metabolic responses, only four genes were found significantly upregulated, and these participate in some amino acids metabolism as well as in oxidative phosphorylation process, whereas the down-regulated genes included participants in multiple pathways such as *alcohol dehydrogenase* and *trifunctional enzyme subunit alpha*. In the immune response, only *MHC class I heavy chain precursor* and *C-C motif chemokine 19 precursors* were significantly upregulated with density, whereas several genes involved in lysosome pathway and others such as Toll-like receptor signaling pathways were on the most downregulated. There was no significantly upregulated gene related to epithelium integrity pathways. Instead, all significantly regulated transcripts had their expression reduced by HD, as *claudin 15* or *beta-tubulin* (*TUBB*).

3.3. Intestinal transcriptomic response - lncRNAs

LncRNAs' expression patterns followed the same tendency as the coding transcripts with 67.7% of lncRNAs that are expressed in both conditions, whereas 29% were expressed exclusively in fish under low density and only 3.3% were exclusive in fish at high density (Fig. 4A). The differentially expressed lncRNAs are mostly downregulated under high density (Fig. 4B), with 87% of those presenting 3 to 10-fold less expression, and 11.3% were downregulated > 10-fold in fish at high density (Fig. 4C). The correlation matrix between the most highly regulated lncRNAs and coding transcripts participating in the selected metabolic pathways, immune and epithelium integrity related pathways are shown in Supplementary Figs. 2S, 3S, and 4S respectively. Although all lncRNAs presented a strong degree of correlation with transcripts in practically all selected pathways, some were more ubiquitous in their correlation presenting a high level of correlation with a high number of coding genes (i.e., Omy100007749, Omy100011326, Omy300024933, and Omy300053540). Overall, the highest percentage of correlation between coding and non-coding RNAs was positive (Fig. 5), identifying a possible direct relationship between the reduction of expression of genes involved in the selected pathways with the reduction of expression of correlated lncRNAs. Nevertheless, the metabolism-related AMPK and insulin signaling pathways presented equal distribution of both negative and positive correlation with lncRNAs, whereas epithelium integrity GAP junction pathway transcripts presented higher negative correlation with lncRNAs (Fig. 5). Herein, to better highlight the likely interplay between coding and lncRNAs, one pathway from each of the three main evaluated groups was chosen. These were for metabolism the fatty acid degradation pathway, for the immune response the Toll-like receptors pathway, and for epithelium integrity the gap junctions pathway (Fig. 6). Notably, genes regulated with fold change > |5| and FDR p-value < 0.05 were highlighted in the pathways, and for each of those, the most correlated lncRNA was selected to identify correlation pattern. In the fatty acid degradation pathway, all the genes that passed the fold change filter were downregulated and so were the corresponding most correlated lncRNAs. These included two of the most regulated genes in metabolic pathways, *aldehyde dehydrogenase* and *trifunctional enzyme* (Table 2). In contrast, in the Toll-like receptors signaling pathway, although all the genes that passed the filter were downregulated in intestines at high density, five presented a negative correlation with the most correlated lncRNAs, and these were *TOLLIP*, *IKKβ*, *p38*, *CD40*, and *IFN-α*. On Gap junction's pathway, all highlighted genes were downregulated in concomitant with the respective most correlated lncRNAs, except *protein kinase A* (*PKA*) which respective lncRNAs was upregulated.

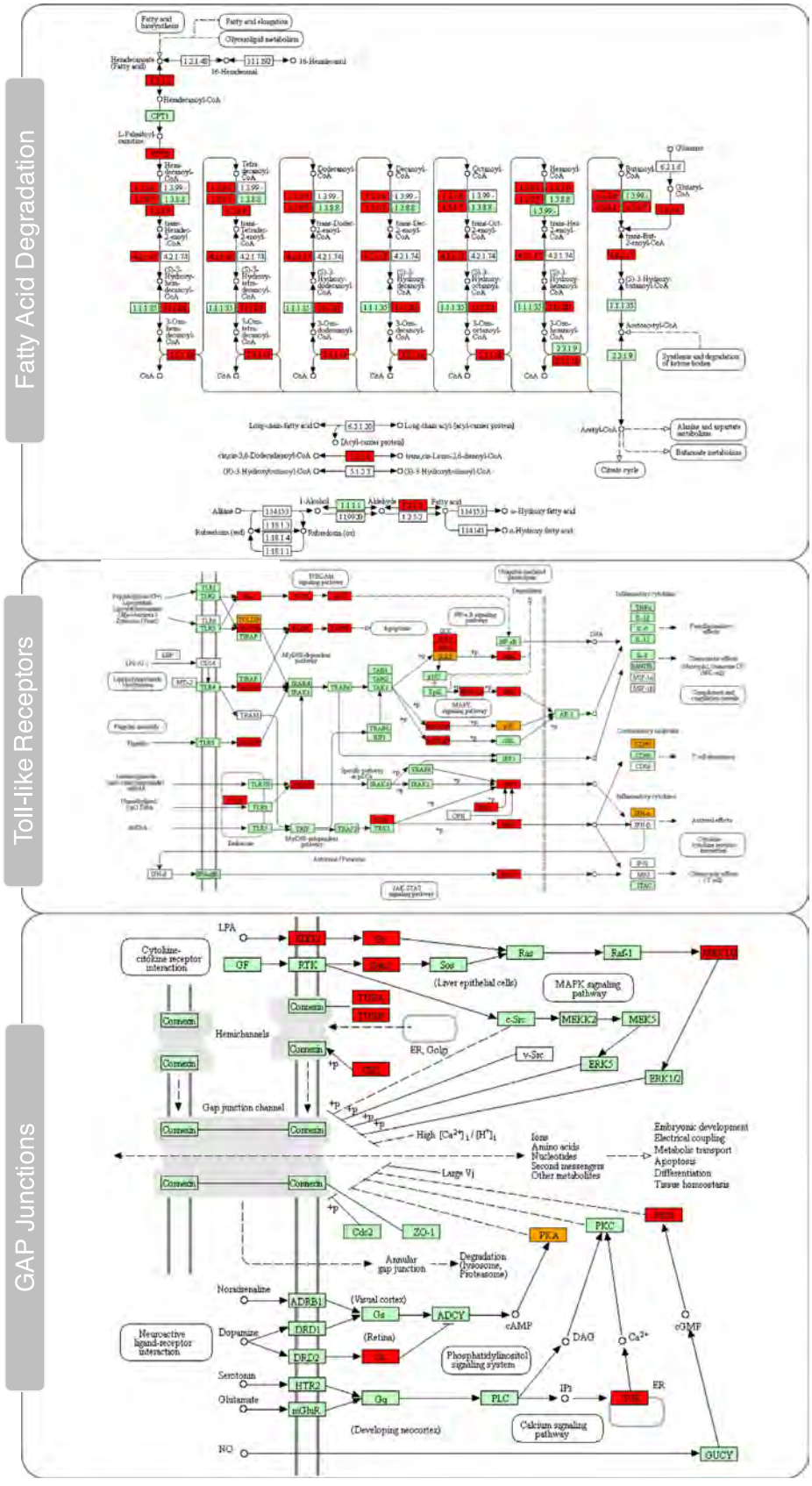


Fig. 6. Differential expressed coding genes and lncRNAs of three example pathways participating in metabolism (Fatty acid degradation), immune response (Toll-like receptors) and epithelium integrity (Gap junctions), retrieved from intestinal transcriptome of rainbow trout stocked at high density for 30 days. All differentially expressed genes were downregulated (see methods for filtering rules), and red boxes indicate that both gene and most correlated lncRNA are down regulated with density, orange boxes indicate that gene is down regulated whereas lncRNA is upregulated, green boxes indicate that genes were not differentially expressed or were with FC < |5|, white boxes indicate genes that were not found in intestinal transcriptome.

Table 1
Summary of *Oncorhynchus mykiss* intestinal sequencing output.

	Low density (LD)	High density (HD)
Reads	15.854.556	17.042.108
Average length (bp)	74.6	75
Nucleotide number (Gb)	1.188.420.610	1.276.456.164
Contigs	33.014	17.586
Average length (bp)	608	488

4. Discussion

Fish stocking density has long been studied as a condition that can be deleterious to fish, disrupting their homeostasis and affecting immunocompetence (Barton, 2002; Ellis et al., 2002). Physiological response to this deleterious condition has been measured in a wide range of species including several biomarkers, and in salmonids, these have been shown to be quite variable and in many cases unreliable to adequately explain in depth stress response in fish (Rebl et al., 2017). The high stocking density used in this study (40 kg m^{-3}) has been considering as an upper limit for stocking density in *O. mykiss* (FAO, 2005–2017), and even higher stocking densities have been reported for being physiological safe for trout (Boujard et al., 2002). This physiological resistance to stocking density in trout explains the constant condition factor observed over the 30 days trial.

To reveal stress response through molecular markers, several studies have been conducted making use of gene expression analysis ranging from qPCR to RNA-seq (Eissa and Wang, 2016; Faught et al., 2016), but only two evaluated the molecular responses in intestines under deleterious conditions (Xia et al., 2013; Gonçalves et al., 2017). Also, when under stimuli, organisms respond with physiological changes, and when these are beyond normal, regulatory reactions are triggered that will affect gene expression in a crucial process for cell and organism biological processes maintenance and stability. To date, the present study is the first to screen intestinal transcriptome response to stocking density – a common factor in aquaculture – evaluating both coding and non-coding transcripts, suggesting for the first time a potential post-transcriptional regulatory action in intestines upon stress. Here we observed that rainbow trout under high stocking density for 30 days present a generalized downregulation of intestine transcriptome, in both coding and non-coding transcripts, evaluated by the expression of the long non-coding RNAs. It was observed a stout downregulation of metabolic pathways, immune-related pathways, and processes related to epithelium integrity and stability, and the transcripts involved in these pathways were found to be highly correlated with identified lncRNAs suggesting a possible post-transcriptional regulation by these ncRNAs during coping with less optimal conditions.

The increase of transaminases activity and glucose levels in plasma (although the later not significant) and reduction of plasma triglycerides level, indicating activation of pathways orchestrating a mobilization and redirection of energy resources (Bonga, 1997; Schreck and Tort, 2016) and with an associated exacerbation of hepatocytes enzymatic activity for energy production, resulting in leaking of marker enzymes in bloodstream (Allison, 2012; Campbell, 2012). These results indicate that although general fish condition was not compromised throughout the experiment, fish were suffering physiological adjustments characteristics from stress coping (Rebl et al., 2017). Intestinal transcriptome response after 30 days at different densities was however profoundly different. Genes that were differentially expressed in intestines of fish stocked at higher density were majorly downregulated, and these results are concomitant with Xia et al. (2013) that found a higher number of downregulated genes and pathways in intestines of Asian seabass exposed to four different challenges (LPS or vibrio injection, fasting and high salinity). As in this study, these authors found

Table 2
Most regulated genes selected for metabolic response, immune response and epithelium integrity.

Contig ID	FC	Annotation	E-value	Pathway
Metabolic response				
Contig0013352	6.1	Creatine kinase B-type	2.6E−15	Arginine and proline metabolism
Contig0018423	26.2	Gamma-glutamyltransferase 5 precursor	4.8E−23	Taurine and hypotaurine metabolism
Contig0009938	5.6	NADH dehydrogenase 1 alpha subcomplex subunit 5	6.5E−21	Oxidative phosphorylation
Contig0005944	5.8	NADH dehydrogenase 1 alpha subcomplex subunit 2	5.7E−19	Oxidative phosphorylation
Contig0045827	−44.7	Aldehyde dehydrogenase, mitochondrial precursor	1.1E−32	Various
Contig0034667	−36.9	Galactocerebrosidase precursor	2.8E−87	Sphingolipid metabolism
Contig0047262	−35.2	Glucosamine–fructose-6-phosphate aminotransferase 1	5.6E−95	Alanine, aspartate and glutamate metabolism
Contig0008232	−34.2	Lissencephaly-1 homolog B	0	Ether lipid metabolism
Contig0041385	−32.2	Alcohol dehydrogenase	1.0E−149	Various
Contig0031350	−30.7	Trifunctional enzyme subunit alpha, mitochondrial precursor, partial	1.2E−151	Various
Immune response				
Contig0024039	55.3	MHC class I heavy chain precursor,	2.4E−55	Various/Phagosome
Contig0006643	10.4	C-C motif chemokine 19 precursor	1.1E−39	Cytokine-cytokine receptor interaction
Contig0021446	−51.9	Lysosomal protective protein precursor	1.6E−147	Lysosome
Contig0057216	−30.2	Gamma-glutamyltranspeptidase 1 precursor	2.0E−17	Arachidonic acid metabolism
Contig0047175	−27.2	Palmitoyl-protein thioesterase 1 precursor	4.7E−33	Lysosome
Contig0031623	−26.9	Caspase-10, partial	4.8E−140	Toll /NOD -like receptor signaling pathway
Contig0054750	−26.1	FYVE finger-containing phosphoinositide kinase, partial	5.9E−06	Phagosome
Contig0001955	−26.1	Procathepsin B precursor	3.0E−47	Lysosome
Contig0009058	−22.1	Stat1–2	2.2E−141	Toll-like receptor signaling pathway
Contig0056160	−22.1	IL-4 receptor-2 precursor	0	Cytokine-cytokine receptor interaction
Epithelium integrity				
Contig0047551	−30.2	Guanine nucleotide-binding protein Gk subunit alpha	6.6E−68	Tight junctions/gap junctions
Contig0020165	−22.1	TUBB	5.5E−98	Gap junction
Contig0050376	−21.1	Serine/threonine-protein phosphatase PP1-gamma catalytic subunit	3.8E−24	Focal adhesion
Contig0033745	−19.9	eEF1A2 binding protein-like	2.3E−6	Focal adhesion
Contig0051451	−17.4	CD166 antigen homolog precursor, partial	1.1E−76	Cell adhesion molecules (CAMs)
Contig0015625	−17.1	Claudin 15	1.0E−78	Tight junctions
Contig0015578	−15.7	Baculoviral IAP repeat-containing protein 4	1.8E−26	Focal adhesion
Contig0014000	−14.1	Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A beta isoform	0	Tight junctions
Contig0038495	−13.6	Band 4.1-like protein 2, partial	5.0E−12	Tight junctions
Contig0023553	−13.3	Ras-related protein Rap-1b precursor	1.3E−79	Focal adhesion

downregulation of genes involved in metabolic pathways or regulation of energy metabolism, suggesting the inhibitory role of the applied challenges on metabolism. However, Liu et al. (2014) showed that short-term confinement and handling has an opposite effect in liver than the one here observed in the intestine.

In contrast with the present study the abovementioned reports that pathways related to metabolism and immunity were upregulated, indicating that the response is stressor and tissue-dependent and so, care should be taken when comparing responses to deleterious conditions (Eissa and Wang, 2016). Although we found upregulation of two subunits of *NADH dehydrogenase 1 alpha subcomplex* – participating in oxidative phosphorylation – and in *creatine kinase B-type* and *gamma-glutamyltransferase 5 precursor* – participating in amino acids metabolism – these were the only genes significantly upregulated in metabolism, leaving no doubts for the overwhelming downregulation of these pathways in intestine (Fig. 1S). The higher expression in liver, of for example *IRS* (insulin receptor substrate), *PI3K* (phosphatidylinositol 3-kinase) and the downregulation of *PPI* (protein phosphatase 1) indicated activation of the insulin signaling pathway in response to short-term confinement (Liu et al., 2014), whereas in our study, when coping with a chronic-type confinement fish intestines respond with down-regulation of this signaling pathway. Immune-related transcripts were also downregulated as previously reported by our group (Gonçalves et al., 2017), and in accordance, with the reported by Yarahmadi et al. (2016) in head kidney of rainbow trout. The same was observed with epithelial integrity and stability related transcripts/pathways, and this reduction of intestinal integrity has been identified as characteristic of both acute and chronic stress (Olsen et al., 2002; Olsen et al., 2005; Sundh et al., 2010). Kelly and Chasiotis (2011) found in rainbow trout's gills that tight junctions' protein-coding genes such as, *claudin* and *ZO-1* were upregulated during exposure to cortisol, whereas in this study, intestinal integrity related pathways were downregulated at high density, highlighting again that responses are stressor and tissue dependent. In addition to the differences observed in the coding transcriptomes, we also found a response associated with non-coding RNAs.

Due to the high correlation observed between transcripts from these pathways and differentially expressed lncRNAs, we here suggest for the first time that these non-coding RNAs might be regulating the response of these pathways in fish under high density, and this might be in a stress-type and tissue-specific mode. Non-coding RNAs have been proved to actively regulate stress responses, homeostasis balance and diseases (Amaral et al., 2013). The knowledge of the regulatory actions of ncRNAs in fish intestines, however, is still far behind, with only one study relating lncRNAs with functional diets modulation (Nuñez-Acuña et al., 2017). Stated grounds for the present study that identifies several lncRNAs with high correlation to some transcripts participating in the selected pathways, but also a few that present a more ubiquitous correlation. These lncRNAs might be of great interest and should be further evaluated in future studies since in mammals it has been found that lncRNAs regulate metabolism, cellular response to stress and epithelium integrity (Lakhotia, 2012; Kornfeld and Bruning, 2014; Wang et al., 2017).

Regarding the later, Xiao et al. (2016) found that a specific lncRNA (*SPRY4-IT1*) in mice, when silenced reduces the expression of tight junction genes (*claudin* and *occluding*) increasing intestine permeability. On the other hand, Xiao et al. (2017) described the regulatory role of another lncRNA (*uc*.173) in intestinal epithelial renewal in mice via reducing the expression of another non-coding RNA (miRNA195). In turn, this evidence indicates that lncRNAs might be regulating the transcriptomic response to suboptimal conditions in fish intestines, and might be the effector of an energy saving strategy (i.e., by shutting down the molecular machinery when at protein level pathways are active), or to maintain the steady stage of transcriptional activity. Either case, as in the cases mentioned above, lncRNAs here reported are potential candidates for future pharmacological strategies for intestine epithelial integrity and stability promotion.

5. Conclusion

Stocking rainbow trout at high density for 30 days induces a strong down-regulatory molecular response in intestines, evidencing a steady decrease in expression of a significant number of genes related to metabolic pathways response, immune response and epithelium integrity and functioning. This is accompanied by significant downregulation of a set of lncRNAs, that as a possible consequence present in their majority positive correlation with coding transcripts. These results suggest a possible regulatory role of these non-coding RNAs in the chronic stress response in fish intestines. We highlighted possible crosstalk of coding genes and most correlated lncRNA in three example pathways. It is relevant to reinforce that each coding gene was found to have a significant correlation with more than one lncRNA, confirming the high complexity of the regulatory roles of these non-coding transcripts, as well as the urgent need to investigate further and uncover the molecular regulatory underpinnings of stress response in fish intestines.

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