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Article

Gene expression profile provides novel insights of fasting-refeeding response in zebrafish skeletal muscle

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Abstract: Recently, fasting has been spotlighted from a healthcare perspective. However, the detailed biological mechanisms and significance by which the effects of fasting confer health benefits are not yet clear. Due to certain advantages of zebrafish, as a vertebrate model widely utilized in biological studies, we used mRNA-sequencing and bioinformatics analysis to examine comprehensive gene expression changes in skeletal muscle tissues during fasting-refeeding. Our results produced a novel set of nutrition-related genes under a fasting-refeeding protocol. We found five dramatically upregulated genes in each fasting (for 24 hours) and refeeding (after 3 hours), exhibiting a rapid response to the provided conditional changes. The assessment of the gene length revealed, the gene set whose expression was elevated only after 3 hours of refeeding had a shorter length, suggesting that nutrition-related gene function is associated with gene length. Taken together, our results from bioinformatics analyses provide new insights into biological mechanisms induced by fasting-refeeding conditions within zebrafish skeletal muscle.

Keywords: fasting; refeeding; skeletal muscle; zebrafish; mRNA-sequencing; gene length

1. Introduction

Inappropriate calorie intake is an important risk factor for cardiovascular disease, and metabolic syndrome [1]. In recent years, fasting and caloric restriction have been attracting a certain attention as a part of health maintenance [1–6]. For example, intermittent fasting, which is a regular energy restriction, can provide health benefits for the prevention or treatment of many chronic diseases, including diabetes, cardiovascular disease, cancer, and neurodegenerative diseases [3]. Previous studies reported the effects of fasting on rodent and human health and lifespan [1,2]. In one study, Goodrick et al. reported that the average lifespan of rats with intermittent feeding from 5 weeks of age increased by up to 80% [7]. However, the biological mechanisms by which fasting provides health benefits still leave many questions being unanswered. In particular, even though skeletal muscle

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consumes significant energy and uses ketones during fasting conditions, the effects of fasting on skeletal muscle are not well understood at the molecular level.

Zebrafish offers several advantages as a model organism that mimics mammalian genetic and physiological responses [8–11]. Zebrafish is a popular animal model because of a simplified maintenance, a rapid growth rate, an ease of genetic manipulation, and fewer ethical restrictions [12]. Fasting and refeeding protocols are commonly used to investigate the biological response of teleosts such as the transition from catabolic to anabolic states [13–20]. Several studies in zebrafish have investigated the comprehensive gene expression response to starvation diets in several tissues [13,21–23]. However, as the molecular mechanisms of a response to a fasting-refeeding protocol remain complex, further investigation at the molecular level using zebrafish is needed. Therefore, in this study we performed mRNA-sequencing and bioinformatics analyses to assess the gene expression profile under fasting-refeeding condition. According to the results, our study provides new insights into biological mechanisms, representing immediate transcriptional responses to the fasting-refeeding approaches in zebrafish; as well as proposes efficient methods of bioinformatics analysis accompanied by mRNA-sequencing analysis of zebrafish skeletal muscle.

2. Materials and Methods

2.1. Zebrafish experiments

Zebrafish experiments in this study were approved by the Animal Care Committee, University of Tsukuba (approval number: 21-406). The overview of this experimental protocol is shown in Figure 1. Three-month-old zebrafish of male/female ratio of approximately 1:1, were purchased from a local vendor (MASUKO; Kuki, Saitama, Japan), and then subjected to an acclimation period of over a month. During the acclimation period, the fish were maintained in a 15 L water tank with water temperature of 28.5 ± 1.0 ℃ and were given standard food for tropical fish (Tetra plankton; Spectrum Brands Japan, Yokohama, Kanagawa, Japan) twice a day every 12 hours. The composition of this standard food is described by the manufacturer as follows: crude protein: 44.0%; crude fat: 11.0%; crude fiber: 2.0%; crude ash: 9.0%; moisture: 8.0%. Each feeding was an amount that the fish could eat within 2 minutes. After the acclimation period, 18–20 fish (gender ratio is 1:1 approximately) were placed in a small 3.5-L water tank with water temperature of 28.5 ± 1.0 ℃ and subjected to a 24-hour acclimation period being supplied with standard food. Next, fish were transferred to a small water tank including 380 mg of the same food. Then, four randomly selected fish (two female and two male) were sacrificed at the following three time points to make each condition. After the feeding, the fish were deprived from food for 24 hours to impose a fasting condition (Fast). Subsequently, other fish were refed using the same amount of food as previously. At 3 and 8 hours following the refeeding, fish were sacrificed to produce conditions of refeeding after 3 and 8 hours (Refed_3h and Refed_8h, respectively; Fig. 1A). In each condition, a muscle tissue was harvested between the anus and caudal tail fin (Fig. 1B), and was placed on ice for further RNA extraction. Average weight (mg) and standard error (SE) for all zebrafish (N = 12, in total) used in this study were 588.7 ± 27.2.

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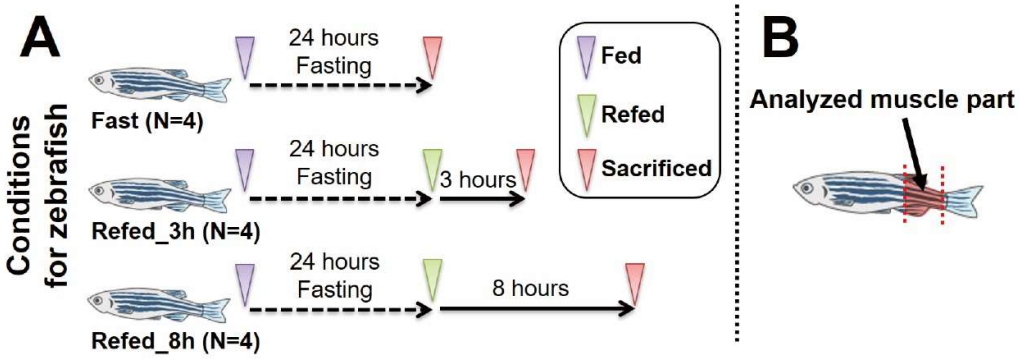

Figure 1. The overview of the experimental protocol. (A) The timing of experimental interventions in each group. (B) The location of the collected skeletal muscle tissue.

2.2. RNA extraction

Total RNA was extracted from the tissues, using RNAiso plus (Cat#9108; Takara Bio, Kusatsu, Shiga, Japan), according to the manufacturer's instructions. The extracted total RNA solution in Milli-Q water was diluted and adjusted to a concentration of 100 ng/μL. Then, 500 ng of RNA was used to make cDNA with the PrimeScript RT Master Mix (Cat#RR036A, Takara Bio), according to the manufacturer's instructions. The cDNA was diluted 10 times by Milli-Q water for further analysis.

2.3. mRNA-sequencing

The mRNA-sequencing (mRNA-seq) was performed to identify the whole gene expression profile in the tissues upon the fasting and refeeding conditions. The mRNA-seq analysis was conducted at the Department of Sports Medicine under the Open Facility Network Office, University of Tsukuba (Tsukuba, Ibaraki, Japan). A total of 12 RNA samples including N = 4 for each condition (i.e. Fast, Refed_3h, and Refed_8h) were examined for integrity using an Agilent RNA 600 Nano Kit (Cat# 5067-1511; Agilent Technologies, Santa Clara, CA, USA) on a Bioanalyzer (Agilent Technologies). After the examination of the RNA Integrity Number, the RNA of all 12 samples was subjected to library preparations for mRNA-seq. Using 1.25 μg of the total RNA from each sample, libraries were created using the NEBNext Ultra II RNA Library Prep Kit for Illumina and the NEBNext Poly(A) mRNA Magnetic Isolation Module (Cat# E7770 and E7490; New England Biolabs, Ipswich, MA, USA), according to the manufacturer's instructions; the final PCR cycle was 12. Concentration and size distributions of the libraries were measured using an Agilent DNA 7500 kit (Cat# 5067-1506; Agilent Technologies) with a Bioanalyzer. All samples were passed for analyses on NGS equipment. The libraries were pooled, and the concentrations adjusted to 1 nM. The pooled libraries were subjected to denaturation and neutralization. Subsequently, the libraries were diluted to 1.8 pM and then applied for an NGS run using NextSeq 500/550 v2.5 (75 cycles) kits (Cat#20024906; Illumina, San Diego, CA, USA) in a NextSeq 500 System (Illumina). The sequencing was performed with paired-end reads of 36 bases. After the sequencing run, FASTQ files were exported, and the basic information of the NGS run data was checked by CLC Genomics Workbench 20.0.4 software (QIAGEN, Venlo, Limburg, Netherlands). In the quality assessment of the reads, a PHRED score over 20 was confirmed for 99.73% of all reads, indicating the success of the run. The read number was approximately 32.1 million to 49.1 million per sample as paired-end reads.

2.4. Bioinformatics analysis

The FASTQ files were mapped to the zebrafish genome (*Danio rerio*; GRCz11) and expression values of all genes were obtained as TPM (Transcripts Per Kilobase Million)

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using the CLC Genomics Workbench software (Table S1). Statistical differential expression analysis was conducted using the "Differential Expression for mRNA-Seq" tool in the CLC. Differentially expressed genes (DEGs) were considered with a false discovery rate (FDR) < 0.05 and a two-fold change cutoff. For the analyses represented in Figure 4, to avoid the possibility of a sample's contamination during the skeletal muscle extraction, genes with a standard deviation greater than 1 (TPM values of each gene were normalized to 1) were excluded in each group. Additionally, genes with an absolute average TPM value of less than 10 were considered as genes with a low expression and excluded from the analysis. A principal component analysis (PCA) plot and Venn diagram were created using the CLC software. Transcripts Per Kilobase Million (TPM) were used as an expression value for figure visualizations. The Database for Annotation, Visualization, and Integrated Discovery (DAVID version 6.8, https://david.ncifcrf.gov/) web tool was used for enrichment analysis with default settings. Public databases of UniProtKB (https://www.uniprot.org/) and ZFIN (https://zfin.org/) were used to investigate function profiles of selected DEGs. The gene length and number of genes used the value annotated in the expression tracks of the CLC software. Heat map with clustering analysis, and box plots were generated using the CLC software, Jupyter Notebook (version 6.1.4), and Python (version 3.8.5). Cluster dendrogram with Ward's minimum variance method was generated using R software (version 4.1.1). Plot graphs were created using GraphPad Prism software (version 9.3.1; (GraphPad, San Diego, CA, USA). The scatter plot was created using Excel (Office 2019; Microsoft, Redmond, WA, USA).

2.5. Statistical analysis

Statistical analyses were performed using GraphPad Prism software (version 9.3.1). To check the normality of the distributions all experimental data without primary analysis of the mRNA-seq data were evaluated with the Shapiro-Wilk normality test. Subsequently, nonparametric tests were used for all data. Comparisons of three groups were performed using Kruskal-Wallis H tests (one-way ANOVA of ranks) followed by a two-stage Benjamini, Krieger, and Yekutieli FDR procedure as a post-hoc test. A *p*-value less than 0.05 was considered to indicate statistical significance.

3. Results

3.1. Overview of gene expression profiling by fasting-refeeding

The mRNA-sequencing analysis was performed in three groups with different feeding conditions: 24-hour fasting (Fast), refeeding after 3 hours (Refed_3h), and refeeding after 8 hours (Refed_8h). The PCA and cluster dendrogram of total gene expressions separated the Refed_3h from the other time points, and the Refed_8h had a similar expression pattern to the Fast condition (Fig. 2A, B). The analysis of differentially expressed genes (DEGs) identified a total of 1,091 expressed genes in three conditions. The Venn diagram (Fig. 2C) exhibited 23 commonly shared DEGs between three comparison groups. In addition, heat map and clustering analyses (Fig. 2D) distinguished four gene-expression clusters that showed following profiles. Clusters 1 (C1) and 3 (C3) were composed of genes with mutually inverse expression patterns between the fasting and the refeeding groups. Similarly, clusters 2 (C2) and 4 (C4) were composed of genes with opposite expression patterns. C2 genes, affected by Refed_3h condition, were downregulated relatively to other two conditions, while C4 genes, affected by Refed_3h, exhibited an inverse expression pattern (Fig. 2E). These results indicate that three groups show different gene expression patterns, and each group drives different biological processes. Furthermore, the correlation of gene expressions within C1 vs. C3 and C2 vs. C4 was mutually inverse.

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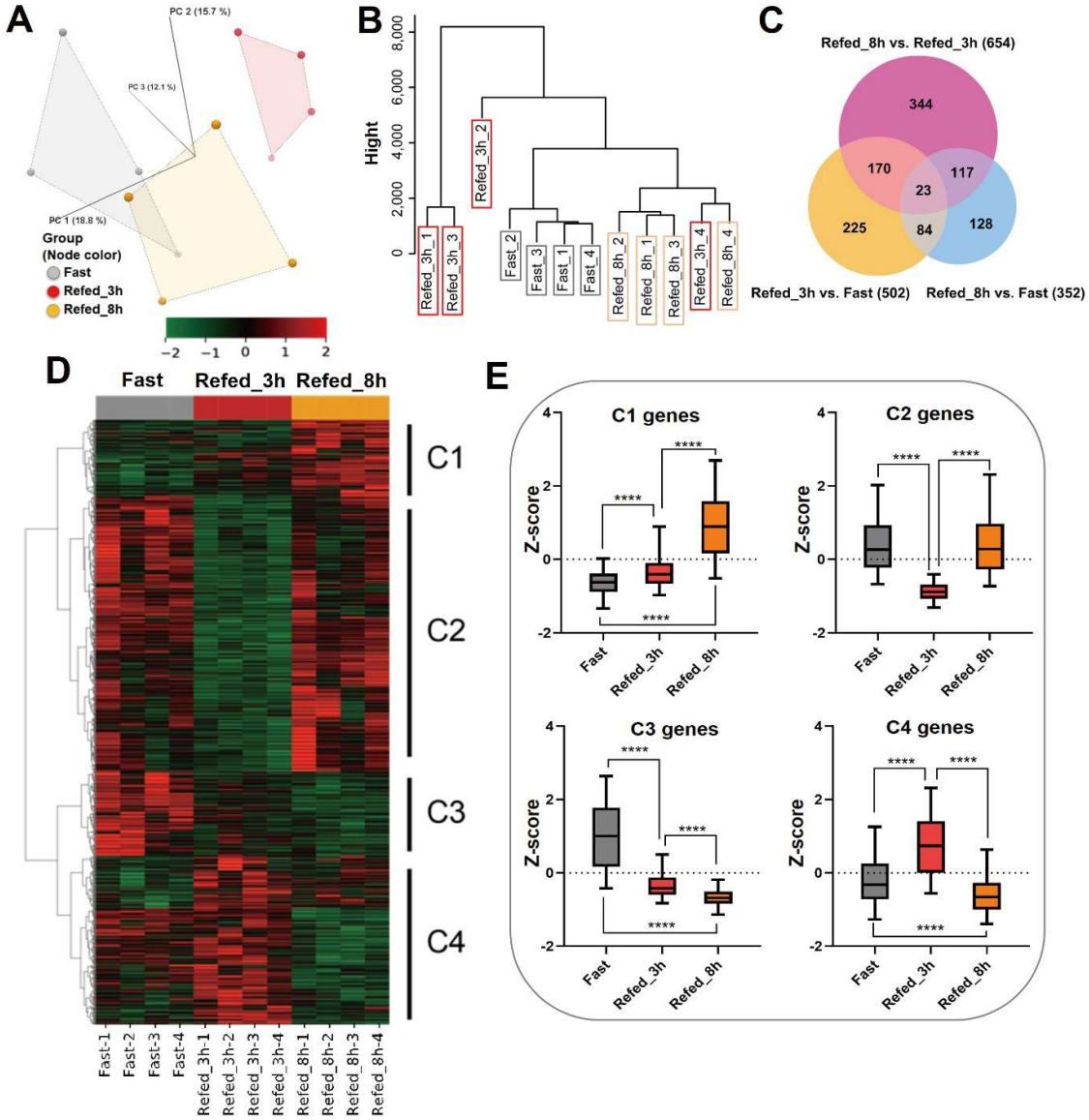

Figure 2. **Gene expression profile of muscle tissues during fasting-refeeding conditions in zebrafish.** (**A**) PCA plot and (**B**) cluster dendrogram depict similarities between samples. (**C**) Venn diagram shows the number of DEGs between three groups (Refed_8h vs. Refed_3h, Refed_3h vs. Fast, and Refed_8h vs. Fast). (**D**) Heat map represents expression of the 1,091 DEGs. Values in the rows are z-scores. (**E**) Box plot shows expression patterns in each cluster on the heat map. The box indicates the first to the third quartile and the line indicates the median. The whiskers show the box with 5–95 percentile. ****p<0.0001.

3.2. Enrichment analysis of DEGs profile affected by the fasting-refeeding

To assess gene sets that may be associated with biological pathways in each cluster (C1–C4) from Figure 2C, we performed pathway analysis using the Kyoto Encyclopedia of Genes and Genomes (KEGG) together with the DAVID web tool. The results showed that the gene set of C1 was enriched in a pathway of fructose and mannose metabolism (Fig. 3A). The gene set of C2 was enriched in the p53 signaling pathway, FoxO signaling pathway, cell cycle, Jak-STAT signaling pathway, progesterone-mediated oocyte maturation, insulin signaling pathway, herpes simplex infection, oocyte meiosis, and cytokine-cytokine receptor interaction. The gene set of C3 was enriched in groups of genes related

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to steroid biosynthesis, metabolic pathways, biosynthesis of antibiotics, primary bile acid biosynthesis, and sulfur metabolism. Finally, the gene set of C4 was enriched in next pathways, such as biosynthesis of antibiotics; biosynthesis of amino acids; metabolic pathways; steroid hormone biosynthesis; cysteine and methionine metabolism; glycine, serine, and threonine metabolism; terpenoid backbone biosynthesis; arginine and proline metabolism; and insulin signaling pathway. In particular, fasting condition of the C2 showed high gene-expression value of the fasting proteolytic system, such as markers of muscle atrophy trim63a, fbxo32, and fbxo25 (Fig. 3B). In addition, p21 (known as cdkn1a), being involved into p53 signaling, was upregulated at Refed_8h but was downregulated at Refed_3h (Fig. 3A, B). We focused on a group of genes whose expression had rapidly increased after 3 hours of the refeeding (Refed_3h); some genes associated with muscle development were enriched, e.g. myog, myod1, and six1b (Fig. 3C). These results suggest that the opposite biological pathways of catabolic and anabolic metabolism were activated in fasting and refeeding conditions.

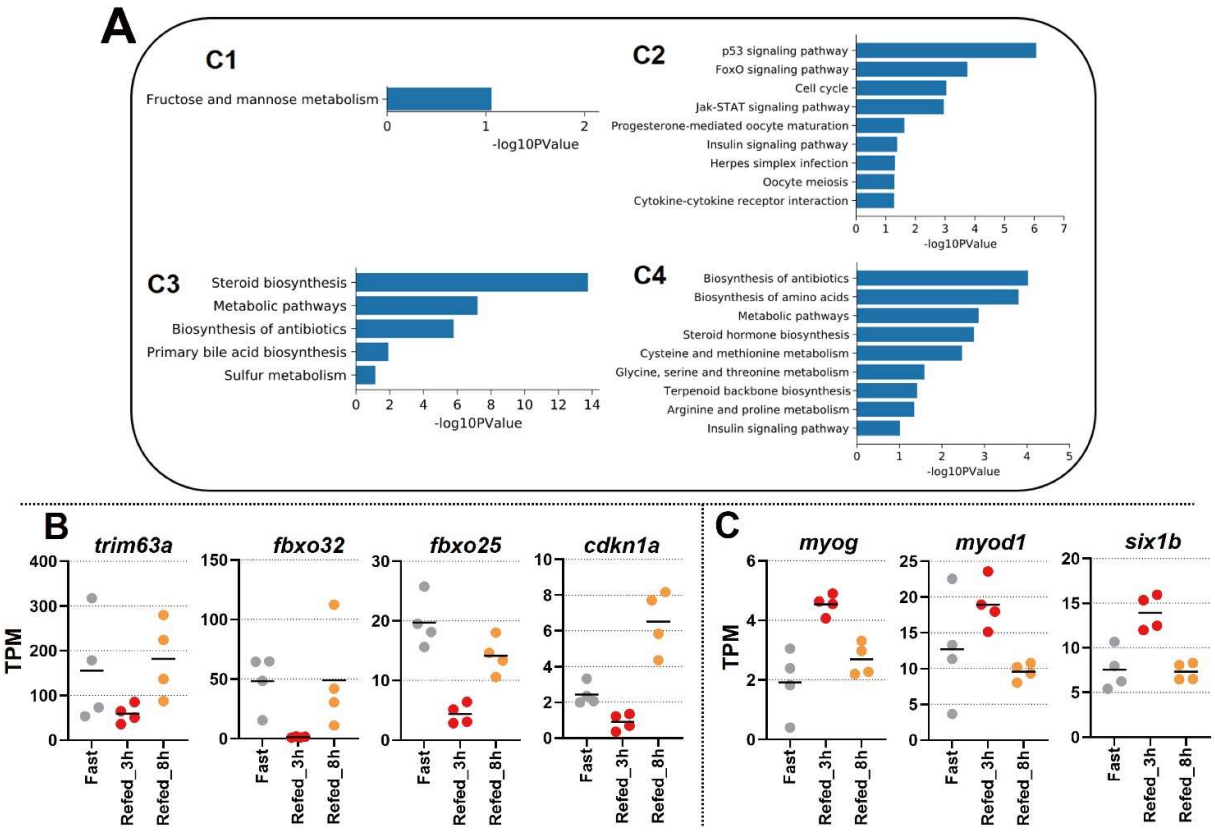

Figure 3. Enrichment analysis in KEGG pathway for gene clusters with confirmed expression values of individual samples. (**A**) Bar plots represent the analysis of significantly enriched pathways in each cluster. (**B**) Individual samples with TPM values of the next genes: *trim63a*, *fbxo32*, *fbxo25*, and *cdkn1a*. The bar indicates average mean. (**C**) Individual samples with TPM values of the next genes: *myog*, *myod1*, and *slx1b*. The bar indicates average mean.

3.3. Identification of differentially expressed genes in zebrafish skeletal muscle affected by fasting-refeeding

Following the mRNA-sequencing, we performed bioinformatics analysis to identify some genes that were affected by fasting-refeeding. Notably, gene clustering revealed that C2 and C4 had an immediate response to both Fast and Refed_3h conditions. Therefore, normalized TPM values of DEGs within C2 and C4, from the Fast vs. Refed_3h

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comparison, were processed by additional algorithmic analyses. Five most expressed genes from each group (Fast and Refed_3h) were visualized by heat map, box plot and scatter plot (Fig. 4A–C). Interestingly, those five genes, which were most upregulated in the Fast group (*pdk2b*, *fbxo32*, *pdk2a*, *klf11b*, and *ulk2*), were downregulated in the Refed_3h group; therefore we named them as "fasting genes". Furthermore, those five genes with dramatic upregulation in Refed_3h group (*mid1ip1l*, *ptgr1*, *pvalb2*, *slc16a3*, and *tecra*) were downregulated in Fast group; therefore we named them as "refeeding genes". Particularly, *pdk2b* was upregulated by 31 folds in the Fast group compared to the Refed_3h group. Similarly to these results, consistent data were obtained from the plot graph (Fig. 4D) evaluating TPM values of an individual sample and gene. Following the expression pattern assessment, we analyzed functions of those genes using publicly available databases (UniProtKB and ZFIN) (Table 1). Next predicted functions, such as glucose homeostasis, autophagy, and ubiquitination were related to the genes affected by the fasting condition. On the other hand, such predicted functions, as lipid and lactate metabolism were related to the genes affected by the refeeding condition. These results suggest the dramatic expression changes of genes related to various metabolic pathways by fasting-refeeding.

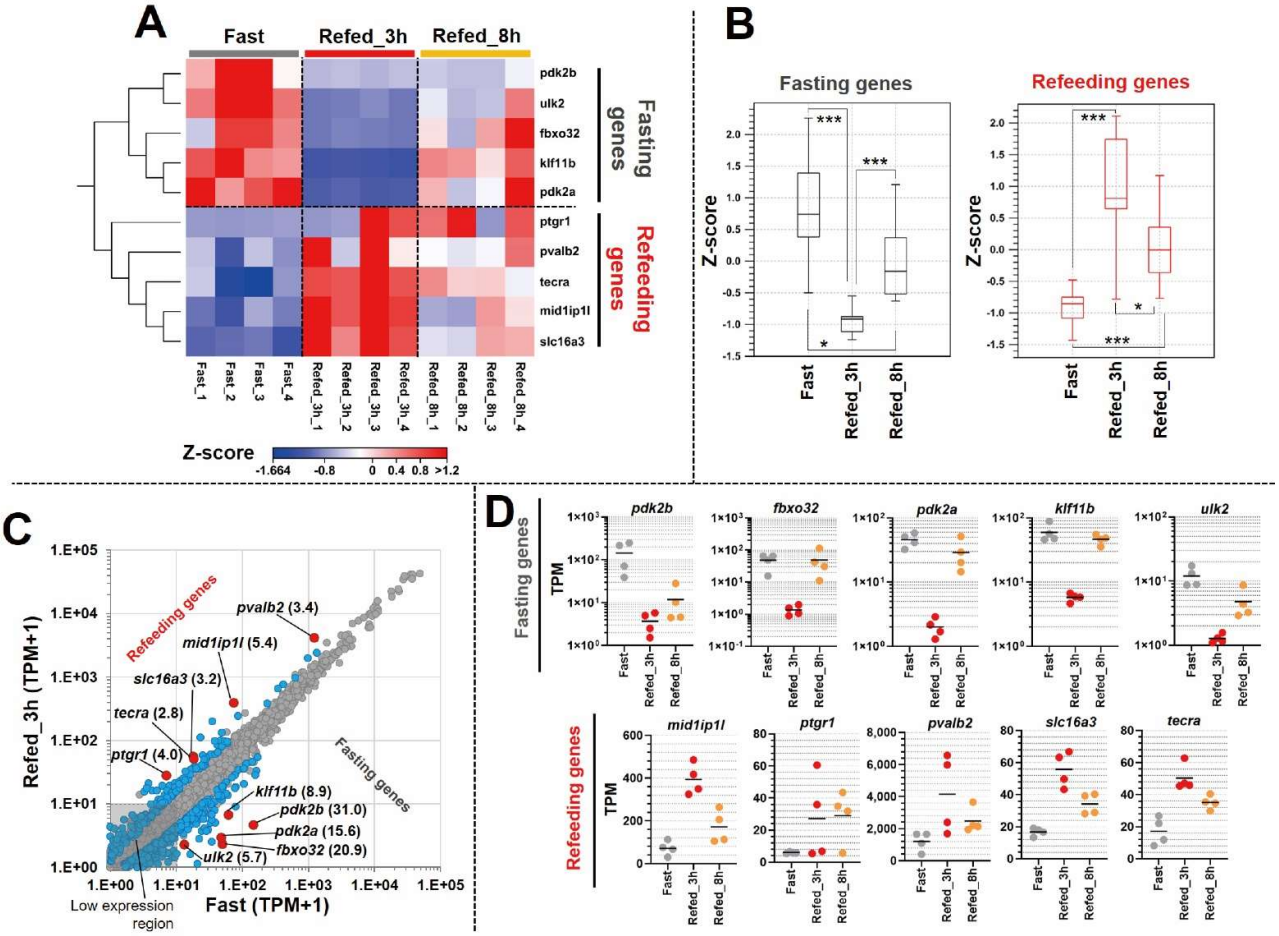

Figure 4. Identification of most affected genes in muscle tissues by fasting-refeeding. (A) Heat map reflects expression values as z-score for 10 genes (5 genes of each condition) identified as most responded genes to fasting-refeeding conditions. Each group (Fast, Refed_3h, Refed_8h) is represented by 4 individual samples. (B) Box plot depicts a z-score of 10 genes in each group. The box indicates the first to the third quartile, and the line indicates the median. The whiskers show the box with 5–95 percentile. (C) Scatter plot reflects averages of TPM+1 values of each gene, shown as red dots. Numbers in parentheses indicate the fold-change between the Fast and Refed_3h groups. Gray

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dots indicate non-DEGs; light blue dots indicate DEGs as FDR *P*-value < 0.05. (D) Plot graph represents TPM values of individual samples with "fasting genes" and "refeeding genes". The TPM values of fasting genes are represented by Log10 notation. The bar indicates average mean. **p*<0.05; ****p*<0.001.

Table 1. Predicted function and fold-change of the top 5 genes in each condition.

Type	No.	Gene symbol	Gene name	Fold-change of Fast vs. Refed_3h	Predicted function
Fasting genes	1	***pdk2b***	*pyruvate dehydrogenase kinase 2b*	31.0	Glucose homeostasis, negative regulation of pyruvate dehydrogenase activity
	2	***fbxo32***	*F-box protein 32*	20.9	Autophagy, protein ubiqutination
	3	***pdk2a***	*pyruvate dehydrogenase kinase 2a*	15.6	Glucose homeostasis, negative regulation of pyruvate dehydrogenase activity
	4	***klf11b***	*kruppel-like factor 11b*	8.9	Regulation of transcription by RNA polymerase II
	5	***ulk2***	*unc-51 like autophagy activating kinase 2*	5.7	Autophagosome assembly, autophagy of mitochondrion
Refeeding genes	1	***mid1ip1l***	*mid1 interacting protein 1, like*	5.4	Regulation of cytoskeleton organization and lipid biosynthetic process
	2	***ptgr1***	*prostaglandin reductase 1*	4.0	Prostaglandin metabolic process
	3	***pvalb2***	*parvalbumin-2*	3.4	Calcium ion binding activity (e.g. relaxation after skeletal muscle contraction)
	4	***slc16a3***	*solute carrier family 16 member 3*	3.2	Lactate transmembrane transporter activity
	5	***tecra***	*trans-2,3-enoyl-CoA reductase a*	2.8	Long-chain fatty acid biosynthetic process

3.4. Novel findings of gene length associated with refeeding

Previous studies have suggested that gene length is involved in life processes and diseases [24–32]. However, it is unclear how genes involved in fasting-refeeding conditions are associated with gene length. Therefore, we analyzed the length distribution of the gene group that responded to each cluster and observed that genes of C4 were significantly shorter than the non-DEGs, genes of C2, and genes of C3 (Fig. 5A). Particularly, the C4 genes had a significantly lower number of exons than non-DEGs, genes of C1, C2, and C3 (Fig. 5C). Additionally, the average intron length of C4 genes was significantly lower than that in non-DEGs, genes of C1, and genes of C2 (Fig. 5E). On the other hand, exon length and length per exon of C4 genes were significantly longer than those in other clusters (Fig. 5B, E). These results indicate that the C4 genes have a long exon length, however, they have a shorter intron and a smaller exon number, resulting in a shorter length of genes affected by refeeding.

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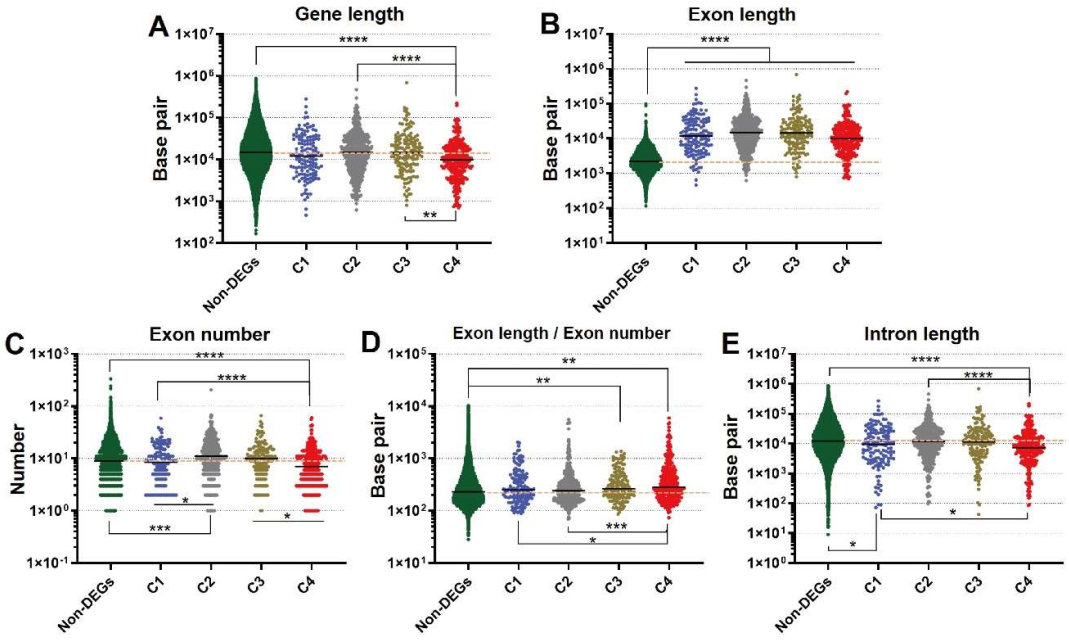

Figure 5. Analysis of gene length, exon length, and exon number in response to refeeding. The graphs show gene length (A), exon length (B), exon number (C), exon length/exon number (D), and intron length (E). The bar indicates the average mean and the orange dashed line shows the average mean of the non-DEGs. **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001, respectively.

4. Discussion

Fasting is spotlighted for potential effectiveness in preventing and treating many chronic conditions, such as diabetes, cardiovascular disease, cancer, neurodegenerative disorders, and delaying aging [1,3–6]. Many studies in rodent models have shown that a fasting protocol has notable effects on longevity, markers for health, stress, metabolic responses, age-related diseases, and tissue regeneration. However, the underlying molecular mechanism of fasting is not fully understood. As a vertebrate organism, zebrafish has become a popular model for biological research because of fewer ethical restrictions, simplified reproduction and maintenance, and rich genomic information. Given that 70% of human genes contain at least one zebrafish ortholog, studies using zebrafish have the potential to deepen the understanding of the detailed role of specific genes in humans. Therefore, this study profiled the transcriptional response to 24 h fasting and subsequent refeeding in zebrafish.

Our results of the enrichment analysis showed that gene expressions associated with the FoxO signaling pathway were increased under the fasting condition. In particular, expressions of *fbxo32* and *trim63a* in cluster 2 were increased as a response to fasting and were decreased as a response to refeeding (Fig. 3A, B). These genes are considered as "atrogenes" that relate to E3 ubiquitin ligase. Especially *TRIM63* and *FBXO32*, which encode MuRF1 and MAFbx respectively, are widely known as master regulators of skeletal muscle atrophy in rodents and human [33-35]. Those genes have reportedly been upregulated by fasting for 10 days in teleosts [36] or for 24 hours in rodents [37]. Moreover, the present study obtained similar results to previous studies; thus, these findings may provide evidence that fasting induces skeletal muscle atrophy. This suggests that zebrafish skeletal muscle is affected by fasting under a similar molecular mechanism as in mammals.

Next, expressions of C2 genes with enrichment in p53 and p21 signaling pathways were upregulated in the fasting condition (Fig. 3A). A previous study in mice showed that p21, being involved into p53 signaling, is upregulated after a 48-hour fasting, suggesting

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that p21 is associated to elevated energy expenditure, depletion of fat stores, and premature activation of protein catabolism in mice muscle [38]. Taken together, zebrafish muscle tissues seem to have p21-related mechanisms similar to those within murine muscles during the fasting condition.

Gene expressions of the cluster 4 were inversely correlated with expressions of C2 genes. Genes involved in myogenesis were enriched in C4. Our results showed that after the 24-hour fasting, *myog* and *myod1* expressions were upregulated in the Refed_3h group (Fig. 3C). Consistently with our results gene *myog* was reported to have an increased expression in zebrafish skeletal muscle 6 hours after refeeding, though there were differences in fasting-refeeding experimental setup (e.g. time point and food type) [23]. However, in the previous study *myod1* was not noted to be upregulated [23]. This discrepancy in results may be caused by differences in experimental conditions. For instance, Seiliez et al. reported that zebrafish received fasting for 72 hours with a following refeeding *ad libitum* and muscle tissues were harvested at 0.5, 2, 6, and 24 h after the refeeding [23]. Our results may have captured a more immediate response because we observed gene expression 3 hours after refeeding. In addition, differences in sampling sites may have affected the results. In order to resolve these differences in the future, it might be necessary to establish detailed and consistent experimental methods.

Our data also showed that *six1b* was upregulated only 3 hours after refeeding (Fig. 3C). There are two orthologs in zebrafish, *six1a* and *six1b*, which have been reported to have important functions in normal muscle formation during embryonic development [39]. Results in this study showed that *six1b* changes immediately during the response to the refeeding. Therefore, an increased expression of *six1b* caused by refeeding may be involved in normal muscle formation of adult zebrafish.

Next, we identified several genes that were dramatically affected by a fasting-refeeding protocol. Totally 10 genes (from C2 and C4) exhibited high expression values and high fold changes within Fast or Refed_3h conditions (Fig. 4A–D). To date, there have been no reports on those genes being affected by fasting and refeeding in zebrafish skeletal muscle. Therefore, these findings provide new insights for researchers in nutrition, internal medicine, etc. to use a zebrafish as a model. For instance, these 10 genes could be utilized in studies as responding markers to new nutrients or compounds for muscle treatment. On the other hand, new cases of people suffering obesity has recently increased, as present days may be considered as an era of satiety. Additionally, sarcopenic obesity among adults is rapidly increasing worldwide [40]. Sarcopenia is a condition characterized by a loss of muscle mass, strength, or physical function, while bearing a potential to induce a frequency of metabolic disorders, cardiovascular disease, and mortality [40]. Although present study used a zebrafish model, we identified an overall mRNA-expression profile and 10 most affected genes. Therefore, our findings could be useful in a treatment development and preventive strategies for the sarcopenic obesity.

Gene length has been reported to be associated with biological regulatory processes. Previous studies suggest that carcinogenesis and other multigenic diseases, embryonic development and neuronal processes are associated with the presence of long genes [24–32]. In addition, bioinformatics analysis indicated that short genes are mainly involved in the immune system and skin formation [41]. These studies lead to the hypothesis that genes with long transcripts are primarily associated with early developmental function, and genes with small transcripts play an important role in routine functions [41]. Though, the relationship between gene length and biological processes is yet to be fully understood, we hypothesize that this mechanism is associated with genes that respond to fasting-refeeding. According to the results, analysis of gene length in the DEGs cluster showed that C4 contained significantly shorter genes (Fig. 5A), and the genes in C4 had less exons (Fig. 5C). The representative genes from C4 may have been efficiently designed during evolution to provide a rapid biological response to refeeding in daily life, which is supported by the hypothesis of the previous study [41]. In addition, chromosomal regions radiate within the nucleus including those chromosomes with a predominant peripheral

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location (heterochromatin), and chromosomes with active genes located inside the nucleus (euchromatin) [42]. We propose that some of the nutritionally regulated short length genes may be located in euchromatin regions with an efficient transcription under nutritional response. Taken together, these results provide new insights into the role gene length has on gene expression.

The effect of dietary restrictions on murine lifespan depends on species, strain, and age at the start of an experiment, with both negative and positive effects reported [43,44]. Therefore, it is necessary to consider whether these differences also occur in zebrafish. Our results were obtained from the single fasting-refeeding response, and it is unclear whether these results will correspond to those after multiple fasting-refeeding approach. Apart from that, the data on gene length were obtained by bioinformatics analysis using a small number of samples, leaving the task of clarifying the actual biological mechanism and significance. Therefore, further *in vitro* and *in vivo* investigation is needed to confirm the molecular findings predicted by this bioinformatics analysis.

5. Conclusions

This study focused on the transcriptome response of fasted zebrafish skeletal muscle tissue. The novel findings in this study, acquired by reliable methods of bioinformatics analysis, provide the use of new gene sets in a zebrafish model with dynamic responses to fasting condition - “fasting genes”, and refeeding condition - “refeeding genes”. Furthermore, this bioinformatics analysis discovered new views on gene length in the regulation of gene expression under the fasting-refeeding conditions. Based on these results, it is expected that zebrafish will provide a new experimental model for fasting-refeeding response and so further significance as an animal model. Future studies may need to continuously assess short- and long-term molecular changes due to fasting or refeeding and link them to positive and negative effects on diseases and other conditions as well as metabolic markers.

Supplementary Materials: The expression data of all genes are available online at (granted after acceptance) in Table S1.

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Institutional Review Board Statement: The study was conducted in accordance with the guidelines (corporation regulation; no. 50, 21 July 2005) of animal experiments by the Animal Ethics Committee of the University of Tsukuba. The approval number was 21-406 (1 June 2021).

Informed Consent Statement: Not applicable.

Data Availability Statement: The mRNA-seq data as FASTQ files and expression browser as table data have been deposited in the "Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/)" under accession number: (It will be released after official acceptance)

Conflicts of Interest: The authors declare no conflict of interest.

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