Studies on Nutritional and Exercise Physiology of Zebrafish

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Studies on Nutritional and Exercise Physiology of Zebrafish

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Abstract

There are few studies on fundamental physiology such as exercise and diet on zebrafish, although research reports on zebrafish with elaborate and abundant biological information are many in the fields of Biochemistry, Genetics and Molecular Biology (including Development). Basic physiology such as exercise and diet is well studied in humans and rodents. However, responses given by exercise to muscles, relationships between diet and obesity, and technologies for improving obesity are still unknown, including differences between species. Therefore, I examined the effects of exercise on skeletal muscle in the field of exercise and muscle physiology, and the effects of the anti-obesity material tea catechins on the zebrafish obesity model which was reported in part in obesity / nutrition metabolism research field in order to collect information on zebrafish exercise physiology and nutritional physiology and deepen insights into this research areas and zebrafish itself.

In examining the effect of exercise on muscle, I examined zebrafish swimming mode in detail, identified the drive part of skeletal muscle, observed the hypertrophy of muscle fibers at muscle fiber level, and clarified exercise quantity‑dependent muscle hypertrophy for the first time. In addition, in order to investigate the molecular mechanism associated with muscle hypertrophy at that time, the gene expression analysis of skeletal muscle was performed, and it became clear that muscle hypertrophic response accompanying exercise is close to that of mammal.

In the study of the relationship between diet and obesity, I analyzed body fat accumulation in zebrafish diet (overfeeding) induced obesity model and analyzed the anti-obesity effect of tea catechins known as anti-obesity food material including its mechanism. Specifically, I quantitatively clarified that body fat which is a problem in obesity, especially visceral fat, accumulates in zebrafish diet induced obesity using X-ray CT for the first time. And it was confirmed that tea catechins have body fat, especially visceral fat, reducing effect similarly to mammals. Furthermore, I analyzed the

anti-obesity mechanism by gene expression analysis of various organs, confirmed the mechanism of anti-obesity due to lipid metabolism enhancement in the liver which is also reported in mammals, and suggested a new anti-obesity mechanism of tea catechins which is the decrease in expression of SOCS 3 in visceral adipose tissue.

The results of these studies suggested that zebrafish has a close relationship with the physiology of rodents and humans in relation to the effect of exercises on muscle, nutritional metabolism related to obesity, and body fat accumulation.

Zebrafish has traditional abundant developmental and genetic biological information and also has features such as high prolificacy and easy genetic manipulation. In this study, the physiological state of exercise and meal of zebrafish where information was limited was clarified. By combining the physiological characteristics of the zebrafish clarified in this study and much biological zebrafish information originally possessed, it is considered that the zebrafish research model can be utilized for development preventive improvement technology of sarcopenia, locomotive syndrome, and metabolic syndrome. I also believe that this will also accommodate social demands for alternatives to animal testing. On the other hands, the research findings on the physiology of zebrafish by this study are the information of zebrafish's Agricultural and Biological Sciences which was insufficient compared to other fish species. The findings concerning exercise, nutritional metabolism, muscle and fat of zebrafish, which is one of the most detailed research findings, are thought to be able to lead to study of aquaculture style and edible part of fish. The multi-faceted research of zebrafish is expected to give further insight into such research and development areas.

Abbreviations

CT	Computed tomography
HU	Hounsfield unit
EF1α	Eukaryotic translation elongation factor 1 alpha 1, like 1
NRF1	Nuclear respiratory factor 1
CS	Citrate synthase
MyoD	Myogenic differentiation 1
MuRF1	Tripartite motif containing 63
PGC1α	Peroxisome proliferator-activated receptor gamma, and coactivator 1 alpha

General Introduction

Zebrafish (*Danio rerio*) are Indian origin which belongs to Cyprinidae (Carp) family and a freshwater tropical Teleoste of about 4 cm in length. This prolific fish is vigorous and easy to keep and reproduce. The history of zebrafish are old, it began to be used in development research in the 1930's (Laale 1977), the development of various genetic engineering techniques applied to zebrafish (for example, ENU (N-Nitroso-N-ethylurea) mutagenesis techniques (Solnica-Krezel 1994, Driever 1996, Haffter 1996), tol2 system of transgenic technology (Kawakami 2004), TALEN/CRISPR-Cas9 of genome editing technology (Huang 2011, Bedell 2012, Friedland 2013, Hwang 2013) and has advanced development sciences. Many papers currently searched for "zebrafish" are also published in journals in development fields. A genome project of zebrafish was started relatively early in the United States (2001), the genome information was already decoded, and many genes have already been identified and analyzed (Howe 2013). In other words, zebrafish can be said to be a very biological information-rich fish.

On the other hand, the research field on 'fish' is the research field of Agricultural and Biological Sciences [Search by Scopus (-2016)　TITLE-ABS-KEY(fish) 437746 hits; Field analysis 1st : Agricultural and Biological Sciences 194115 hits (44%), 2nd : Biochemistry, Genetics and Molecular Biology 109333 hits (25%), including duplication]. For example, as fishes with a lot of research reports, salmon in which a high aquaculture technique is developed can be mentioned, but research reports on salmon mainly include research reports on Agricultural and Biological Sciences [Search by Scopus (-2016) : TITLE-ABS-KEY(Salmon) 36859 hit; Field analysis 1st: Agricultural and Biological Sciences 19002 hit (52%), 2nd: Biochemistry, Genetics and Molecular Biology 8223 hit (22%), including duplication]. On the other hand, research reports on zebrafish with elaborate and abundant biological information are many in the fields of Biochemistry, Genetics and Molecular

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Biology (including Development) [Search by Scopus (-2016) TITLE-ABS-KEY(zebrafish) 30397 hits; Field analysis 1st: Biochemistry, Genetics and Molecular Biology 19730 hits (65%), 2nd: Medicine 9090 hits (30%), 3rd: Agricultural and Biological Sciences 5160 hit (17%), including duplication], and conversely there are few studies on fundamental physiology such as exercise and diet.

Basic physiology such as exercise and diet is well studied in humans and rodents. However, responses given by exercise to muscles, relationships between diet and obesity, and technologies for improving obesity are still unknown, including differences between species.Regarding the effect of exercise on muscle, Rodnick et al. (1989) studied the relationship between running distance (total work) and skeletal muscle mass during voluntary no-load wheel running for 6 weeks by juvenile Sprague–Dawley rats and reported hypertrophy of the soleus muscle but no dose response. Ishihara et al. (1998) showed a dose–response relationship between running distance (total work) and skeletal muscle mass (plantaris) during voluntary load-wheel running for 8 weeks by juvenile Sprague–Dawley rats. However, these studies did not investigate the effects of changing exercise duration and did not regulate exercise quantity because they studied voluntary wheel running. In contrast, treadmill studies in mice have regulated exercise quantity but have not examined the relationship between exercise quantity (duration/day and overall duration) and skeletal muscle mass (Kemi et al. 2002; Jeneson et al. 2007). Regarding the relationship between diet and obesity, and the technologies for improving obesity, Oka et al. (2010) demonstrated the usefulness of a zebrafish model of DIO that shares common pathophysiological pathways with mammalian obesity. DIO zebrafish have also been used to validate the antiobesity effects of natural products (Tainaka et al. 2011). However, body fat mass, its distribution, and its change, which are important in studying obesity, were unknown.

The cause of these problems was considered to be technically difficult to load exercise with high accuracy and to detect changes in body fat mass and its distribution in small aquatic organisms such as zebrafish. Therefore, I examined the effects of exercise on skeletal muscle in the field of exercise and muscle physiology (Part I), and the effects of the anti-obesity material tea catechins on the zebrafish obesity model which was reported in part in obesity/nutrition metabolism research field (Part II) in order to collect information on zebrafish exercise physiology and nutritional physiology and deepen insight into this research areas and zebrafish itself.

Part I

Exercise Quantity‑Dependent Muscle Hypertrophy in Adult Zebrafish (*Danio rerio*)

Abstract

Exercise is very important for maintaining and increasing skeletal muscle mass, and is particularly important to prevent and care for sarcopenia and muscle disuse atrophy. However, the dose–response relationship between exercise quantity, duration/day, and overall duration and muscle mass is poorly understood. Therefore, I investigated the effect of exercise duration on skeletal muscle to reveal the relationship between exercise quantity and muscle hypertrophy in zebrafish forced to exercise. Adult male zebrafish were exercised 6 h/day for 4 weeks, 6 h/day for 2 weeks, or 3 h/day for 2 weeks. Flow velocity was adjusted to maximum velocity during continual swimming (initial 43 cm/s). High-speed consecutive photographs revealed that zebrafish mainly drove the caudal part. Additionally, X-ray micro computed tomography measurements indicated muscle hypertrophy of the mid-caudal half compared with the mid-cranial half part. The cross-sectional analysis of the mid-caudal half muscle revealed that skeletal muscle (red, white, or total) mass increased with increasing exercise quantity, whereas that of white muscle and total muscle increased only under the maximum exercise load condition of 6 h/day for 4 weeks. Additionally, the muscle fiber size distributions of exercised fish were larger than those from non-exercised fish. I revealed that exercise quantity, duration/day, and overall duration were correlated with skeletal muscle hypertrophy. The forced exercise model enabled me to investigate the relationship between exercise quantity and skeletal muscle mass. These results open up the possibility for further investigations on the effects of exercise on skeletal muscle in adult zebrafish.

Introduction

Reduced skeletal muscle mass results in muscle weakness and a limited ability to support the body. Age-related sarcopenia and disuse muscle atrophy reduce exercise capacity as muscle mass decreases. Indeed, exercise is very important for maintaining and increasing skeletal muscle mass and is particularly important for preventing and treating sarcopenia and disuse muscle atrophy (Borst 2004; Melov et al. 2007; Reid and Fielding 2012; Bloomfield 1997). However, the dose–response relationship between exercise quantity (duration/day and overall duration) and muscle mass is poorly understood. I assume that the reason for this is a lack of appropriate, high-precision animal models.

Rodnick et al. (1989) studied the relationship between running distance (total work) and skeletal muscle mass during voluntary no-load wheel running for 6 weeks by juvenile Sprague–Dawley rats and reported hypertrophy of the soleus muscle but no dose response. Ishihara et al. (1998) showed a dose–response relationship between running distance (total work) and skeletal muscle mass (plantaris) during voluntary load-wheel running for 8 weeks by juvenile Sprague–Dawley rats. However, these studies did not investigate the effects of changing exercise duration and did not regulate exercise quantity because they studied voluntary wheel running. In contrast, treadmill studies in mice have regulated exercise quantity but have not examined the relationship between exercise quantity (duration/day and overall duration) and skeletal muscle mass (Kemi et al. 2002; Jeneson et al. 2007).

Zebrafish (*Danio rerio*) are vertebrates, and the structure and function of their organs and tissues are similar to those of humans. Zebrafish have recently been used as animal models in biomedical research (Lieschke and Currie 2007; Kishi et al. 2009; Best and Alderton 2008; Kishi 2014; Patton et al. 2014; Santoriello and Zon 2012) because of their advantages, such as ease of genetic manipulation and the low cost of breeding and testing. Studies on exercise and skeletal

muscle in zebrafish began to increase in the early 2000s. For example, suppressing myostatin in zebrafish causes a double-muscle effect. Aging in zebrafish causes skeletal muscle atrophy, decreases physical ability (Gilbert et al. 2014), and increases the accumulation of oxidized proteins in skeletal muscle (Kishi et al. 2003). It has also been found that exercise training in zebrafish enhances body growth (Palstra et al. 2010), leads to white muscle fiber hypertrophy (Palstra et al. 2014), and enhances expression of genes related to skeletal muscle production, myogenesis, and energy metabolism (Palstra et al. 2010; Palstra et al. 2014; McClelland et al. 2006). These reports suggest that skeletal muscle production, changes with aging, and the effects of exercise in zebrafish are similar to those in mammals. Furthermore, Lin (2012) and Hirata (2009) reported zebrafish as a useful animal model to study muscle diseases and drug discovery. Moreover, fish have been proposed to be an exceptional model for investigating vertebrate skeletal muscle function because their skeletal muscle is composed of red (slow) muscle and white (fast) muscle, as in mammals; but the muscle is separated into discrete red and white areas (i.e., zebrafish: Fig. 1), unlike in mammals (Rome 2005). Different muscle types or different muscle fiber types can be studied more easily. Therefore, zebrafish would be useful for studying the quantitative relationship between exercise and red/white skeletal muscle mass. However, the previous zebrafish studies did not investigate the details of the relationship between exercise quantity and skeletal muscle mass. In addition, the mitochondrial gene expression response to exercise in zebrafish has been suggested to differ from that of mammals (Lemoine et al. 2010a); however, the expression pattern of genes involved in muscle catabolism and muscle cell proliferation and differentiation are unknown in zebrafish during acute exercise.

Here, I identified the area of skeletal muscle in zebrafish that generates the propulsive force for swimming by analyzing swimming with a high-speed camera and validated the area of skeletal

muscle hypertrophy using X-ray micro computed tomography (CT) measurements. I investigated the effect of exercise duration/day and overall exercise duration on skeletal muscle to reveal the relationship between exercise quantity and skeletal muscle mass. I also investigated skeletal muscle gene expression levels during acute exercise training to confirm the muscle response to exercise intensity and the maximum velocity during continual swimming.

Materials and methods

Animals

Adult male zebrafish (*Danio rerio*) were offspring of fish purchased from a local pet supplier (Meito-Suien Co., Ltd., Remix, Nagoya, Japan). All fish were kept and raised at approximately 28°C under a 14-h light and 10-h dark cycle, and water quality conditions were maintained according to The Zebrafish Book (Westerfield 2007). The fish were concurrently spawned from the same parent in each experiment described below and were 6–7 months of age.

Exercise setup

The exercise setup for the zebrafish was a 320 L Brettstyle recirculating swim flume, which was modified from the Personal-Tank PT-70S (West Japan Fluid Engineering Laboratory Co., Sasebo, Japan). This setup has three swim courses (all 70-cm long, 10-cm wide, and 30-cm high) for exercise and a non-flow bypass tank (1.7 L) as a non-exercise control. Each swim course or non-flow bypass tank has a maximum capacity of ten fish. Photoperiod and water were maintained under breeding conditions.

Experimental design

Experiment 1

Fish were weighed under anesthesia with 0.0075% (w/v) tricaine (Sigma-Aldrich, St. Louis, MO, USA) and allocated into two groups (n = 12/group) with similar bodyweights. One group (exercised group) was transferred to the swim courses, and the other group (non-exercised group) was transferred to the non-flow bypass tanks. The exercised group was exercised 6 h/day, 5 days/week for 4 weeks. Swimming form was studied using a high-speed digital camera

EX-ZR1000 (Casio Computer Co., Tokyo Japan) from the dorsal side at 240 fps to observe propulsive movement. Flow velocity was adjusted for the maximum velocity at which the fish could swim continuously (initial 43 cm/s) and was readjusted once/week. All fish were fed a full diet twice daily. The diet was composed of 60% Otohime B2 (Marubeni Nisshin Feed Co., Tokyo, Japan) and 40% gluten (Wako Pure Chemical Industries, Osaka, Japan) (Zang et al. 2011). The day after the final exercise day, the fish were euthanized with excess anesthetic; their body weight was measured; and they were gutted by laparotomy. A cross-sectional area of the skeletal muscle on each side was estimated using X-ray micro-computed tomography (CT). Then, the sample was preserved in 10% neutral buffered formalin solution (Wako Pure Chemical Industries).

Experiments 2 and 3

Fish were allocated into two groups (n = 8/group) with similar body weights. One group (exercised group) was transferred to the swim course, and the other group (nonexercised group) was transferred to the non-flow bypass tank. The exercised group was exercised 6 h/day, 5 days/ week for 2 weeks during experiment 2 and for 3 h/day, 5 days/week for 2 weeks during experiment 3. Flow velocity was adjusted to the maximum velocity at which the fish could swim continuously (43 cm/s) and was readjusted once/week. All fish were fed the full diet twice daily, as in experiment 1. The day after the final exercise day, the fish were euthanized with excess anesthetic, their body weight was measured, and they were preserved in 10% neutral buffered formalin solution.

Experiment 4

Twenty fish were divided randomly into exercised and non-exercised groups (n = 10/group). After the fish in the exercised group swam at 21.5 cm/s for 15 min (acclimation), swimming speed

was increased to 43 cm/s (maximum velocity at which the fish could swim continuously) for 2 h 45 min. Fish in the non-exercised group were transferred to the non-flow bypass tank. All fish were euthanized with excess anesthetic and iced immediately after training. Whole bodies were preserved in RNAlater (Sigma-Aldrich) after laparotomy and stored at 4°C until the gene expression analysis.

Skeletal muscle area measurement by X‑ray micro‑CT

Dissected zebrafish were fixed in a stretched position on a sample holder. The X-ray micro-CT scan was performed with an in vivo System R_mCT 3D micro-CT scanner (Rigaku Corp., Tokyo, Japan). The settings were: voltage 90 kV, current 100 µA, magnification 4×, slice thickness (scanning width) 50 µm, and shooting time 17 s. The images were viewed and reconstructed with i-View type R software (J. Morita Mfg., Kyoto, Japan). The CT images were visualized and analyzed using dedicated CT Atlas Metabolic Analysis ver. 2.03 software (Rigaku). The Hounsfield unit (HU) value of the muscle, which is a high moisture tissue, was considered to be near the HU of water (HU = 0) and between the HU of fat tissue (−350.0 to −145.0, according to the manufacturer's instructions) and the HU of bone (>224.6 to detect the bone signal). X-ray micro-CT can measure the volume of total tissue, fat tissue, or bone within the measurement area based on the difference in X-ray transmittance among tissues. Therefore, I measured of skeletal muscle volume using X-ray micro-CT because the samples consisted mainly of muscle, fat, and bone. Skeletal muscle cross-sectional area was measured on the axial section containing the third abdominal vertebra as the mid-cranial half muscle (Fig. 2, line A) and the axial section containing the posterior end of the anal fin junction as the mid-cranial half muscle (Fig. 2, line B), which were searched in the 3D X-ray micro-CT data.

Skeletal muscle morphometrics analysis

The morphometrics analysis was done by the same person, who was blinded to the study design. Red muscle, white muscle, and these muscle fibers were observed in hematoxylin–eosin (HE)-stained cross-sections of the caudal half muscle (Fig. 2, line B) using a BZ-9000 (Keyence Co., Osaka, Japan) microscope. The cross-sectional area of the muscle or muscle fiber was measured using the manual area definition and BZ-II (Keyence) image analysis software. All skeletal muscle area and muscle fiber size measurements were made in the whole epaxial quadrant (Fig. 1b). The total numbers of fibers analyzed in each muscle sample were 106–272 (red muscle) and 441–886 (white muscle).

Gene expression analysis

Skeletal muscle tissues were collected from the caudal half of the muscle and placed in RNAlater to extract the RNA. Total RNA was extracted using the RNeasy lipid tissue mini kit (Qiagen K.K., Tokyo, Japan) according to the manufacturer's instructions. cDNA was synthesized using the High-Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA, USA). Quantitative real-time polymerase chain reaction (PCR) was performed on the cDNA samples using the TaqMan Fast Universal PCR Master Mix (Applied Biosystems) or the Fast SYBR Green Master Mix (Applied Biosystems) and the ABI Prism 7500 Fast Real-Time PCR System (Applied Biosystems) in accordance with the manufacturer's instructions. TaqMan gene expression assays included: eukaryotic translation elongation factor 1 alpha 1, like 1 [EF1α: Dr03432748_m1 (GenBank: L47669)], nuclear respiratory factor 1 [NRF1: Dr03074214_m1 (GenBank: AL590150)], citrate synthase [CS: Dr03434061_m1 (GenBank: CR381531)], myogenin [Dr03138081_m1 (GenBank: AF202639)], myogenic differentiation 1 [MyoD: Dr03138243_g1 (GenBank:

BC114261)], F-box protein 32 [atrogin1: Dr03151496_m1 (GenBank: BC052112)], and tripartite motif containing 63 [MuRF1: Dr03193823_s1 (GenBank: BC071428)]. The SYBR Green primer sets were: peroxisome proliferator-activated receptor gamma and coactivator 1 alpha [PGC1α (GenBank: AY998087), forward primer (5′–3′): TGAGGAAAATGAGGCCAACT, reverse primer (3′–5′): AGCTTCTTCAGCAGGGAAGG]. Baseline and threshold were set manually in accordance with the manufacturer's instructions. Relative mRNA expression levels were determined using EF1α as an endogenous standard.

Statistical analyses

All data are presented as means ± standard errors unless otherwise indicated. The normality of all data was tested using the Kolmogorov–Smirnov test (with Lilliefors' correction) for the histogram analysis of muscle fiber crosssectional area or the Shapiro–Wilk test for all other data. Comparisons of the muscle cross-sectional area results in experiment 1, the mean cross-sectional muscle fiber area in experiment 1, and the histogram analysis of the muscle fiber cross-sectional area in experiments 1–3 between the non-exercised and exercised groups were analyzed using the Mann–Whitney U test because the data were not normal. All other normal data were analyzed with Student's t test. The increase in the skeletal muscle ratio to test the dose-dependency between exercise quantity and skeletal muscle hypertrophy was analyzed using the Jonckheere–Terpstra test for trends. Statistical analyses were performed using IBM SPSS Statistics ver. 23 (IBM Corp., Armonk, NY, USA). A p value <0.05 was considered significant.

Results

Water flow, food consumption, and body weight

Water flow during all exercise training was increased 10% each week, except week 4 in experiment 1 (Table 1). All fish were able to complete the exercise training. Food consumption of the exercised training group increased by 45% during experiment 1, by 23% during experiment 2, and by 14% during experiment 3 compared with that in nonexercised fish, although no difference in body weight was detected between the exercised and non-exercised groups (Table 2).

Identifying the maximum skeletal muscle driving area during swimming

I defined the driving area as the maximum bent position of the body, and considered that this area generated most of the propulsive force for swimming. The high-speed sequential photography revealed that zebrafish drove mainly from their caudal half muscle compared with the cranial half when swimming quickly (Fig. 3). The ratio of the midcaudal half skeletal muscle cross-sectional area (Fig. 2, line B) to the mid-cranial half skeletal muscle cross-sectional area (Fig. 2, line A) was higher in exercised fish than that in non-exercised fish (Fig. 4).

Effect of exercise term and duration on skeletal muscle area and fiber size

Red, white, and total muscle cross-sectional areas measured at the ends of experiments 1–3 are shown in Table 2. Red muscle cross-sectional areas of exercise-trained fish in experiments 1–3 were larger than those in non-exercised fish. White and total muscle cross-sectional areas in experiment 1 were larger than those in non-exercised fish. The skeletal muscle ratios (red, white, and total) increased in each experiment as calculated from muscle cross-sectional area of individual fish in the exercised group divided by mean muscle cross-sectional area in the non-exercised group (Fig.

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5). The red, white, and total muscle ratios increased in response to increased exercise quantity, low: experiment 3 (2 weeks, 3 h), mid: experiment 2 (2 weeks, 6 h), high: experiment 1 (4 weeks, 6 h). Mean cross-sectional red and white muscle fiber sizes in experiments 1–3 are shown in Fig. 6. In experiment 1, mean cross-sectional red muscle fiber sizes were 554.7 ± 24.5 and 705.0 ± 31.6 μm^2 in non-exercised and exercised fish (Fig. 6a), respectively, whereas white muscle fiber sizes were 1543.0 ± 28.6 and 1661.2 ± 40.3 μm^2 in non-exercised and exercised fish, respectively (Fig. 6b). Both red and white muscle fiber sizes from exercised fish were larger than those from non-exercised fish. In experiment 2, mean cross-sectional red muscle fiber sizes were 478.4 ± 23.5 and 593.7 ± 16.2 μm^2 in non-exercised and exercised fish, respectively (Fig. 6c), whereas white muscle fiber sizes were 1532.8 ± 60.6 and 1544.7 ± 59.2 μm^2 in non-exercised and exercised fish, respectively (Fig. 6d). Red muscle fibers from exercised fish were larger than those from non-exercised fish. In experiment 3, mean cross-sectional red muscle fibers sizes were 496.5 ± 18.9 and 604.7 ± 17.5 μm^2 in non-exercised and exercised fish, respectively (Fig. 6e), whereas white muscle fiber sizes were 1451.4 ± 36.2 and 1502.5 ± 51.9 μm^2 in nonexercised and exercised fish, respectively (Fig. 6f). Only red muscle fibers from exercised fish were larger than those from non-exercised fish. The cross-sectional red and white muscle fiber size distributions in experiments 1–3 are shown in Fig. 7. The muscle fiber size distributions of exercised fish, other than white muscle in experiment 2 (Fig. 7d), were larger than those from non-exercised fish.

Effect of exercise training on gene expression in skeletal muscle

The acute mRNA expression response was assessed in genes encoding a regulator of mitochondrial biogenesis, function, and activity (PGC1α, NRF1, and CS); a muscle differentiation regulator (MyoD); a muscle development regulator (myogenin); and muscle-specific ubiquitin

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ligases (atrogin1 and MuRF1) during the single exercise examination in experiment 4 (Fig. 8). All

mRNA levels in exercised fish were significantly higher than those in non-exercised fish.

Discussion

Observing the swimming form of zebrafish showed that they drove the caudal-half of the skeletal muscle to generate high speeds during swimming. The X-ray micro-CT measurements revealed muscle hypertrophy of the midcaudal half part compared with that of the mid-cranial half part. These findings show that skeletal muscle in the caudal half hypertrophies as exercise duration per day and overall duration increases over time in exercised fish (experiments 1–3) compared with that in non-exercised fish (Fig. 5). Experiments 1–3 revealed skeletal muscle hypertrophy with increasing duration/day and term, which was considered dose-dependent exercise quantity. White muscle also hypertrophied with more exercise and increased significantly with duration/day and term in this study. Additionally, the gene expression patterns observed during a single exercise bout suggested that exercise intensity in this study was sufficient to affect skeletal muscle. Therefore, I demonstrated for the first time in zebrafish that exercise quantity dose-dependently increased skeletal muscle hypertrophy. Skeletal muscle hypertrophy is an adaptation to the quantity of exercise that also occurs during human exercise training (Konopka and Harber 2014). No forced exercise study has shown that increasing exercise quantity increases skeletal muscle mass within 4 weeks. Therefore, I suggest that zebrafish is a promising model to investigate the effect of exercise on skeletal muscle and to develop technologies, medicine, and foods that will help individuals maintain and increase skeletal muscle mass.

In this study, I determined that the caudal half skeletal muscle contracted and bent the body to generate the main propulsive force for swimming, which was analyzed with a high-speed camera to ensure that exercise quantity was dose-dependently associated with skeletal muscle hypertrophy. Hypertrophy of skeletal muscle is a response to a new load and the need for increased poster output to maintain position against increased flow in the case of fish. In mammals, the soleus and plantaris

muscles respond to exercise and are often used to investigate the relationship between exercise quantity and skeletal muscle mass because they drive walking and running. Most mammalian skeletal muscle studies isolated muscle and weighed each part. However, no study has isolated and weighed fish skeletal muscle because it has a different structure. Meulen et al. (2006) measured skeletal muscle cross-sectional area to study skeletal muscle hypertrophy during exercise in juvenile zebrafish. In their report, exercise quantity-dependent skeletal muscle hypertrophy was not shown clearly because growth rate in juvenile zebrafish is affected by skeletal muscle hypertrophy. Other zebrafish studies measured body weight (Palstra et al. 2010) or skeletal muscle fiber size (Palstra et al. 2014) to investigate the relationship between exercise and skeletal muscle mass. Accordingly, it is unclear how much exercise causes dose-dependent skeletal muscle hypertrophy. I determined the skeletal muscle area driving high-speed swimming. Each fish has a respective swimming mode, as the suffix "-form" (e.g., thunniform) refers to the type of movement and not to the body form. For example, the mode is classified based on the type of muscle used, such as caudal muscle or the trunk (e.g., anguilliform, subcarangiform, carangiform, thunniform, or ostraciiform) to the type using the dorsal, anal, or pectoral fin (e.g., amiiform, balistiform, tetraodontiform, gymnotiform, rajiform, diodontiform, or labriform) for propulsive movement (Lindsey 1978). Although zebrafish are assumed to be the caudal type, such as anguilliform, subcarangiform, carangiform, thunniform, or ostraciiform, there was little detailed knowledge of zebrafish swimming mode until now. Therefore, I observed high-speed consecutive photographs of zebrafish swimming (Fig. 3) and the appearance (Fig. 2) of zebrafish with reference to Lindsey (1978), and considered that zebrafish are carangiforms because they have major characteristics of carangiforms, such as wavelength/body length >1.0; wavelength visible on body <0.5; amplitude/body length: undulations confined to posterior 1/3; body shape: mass concentrated anteriorly and peduncle quite narrow; and causal fin: posterior margin

notched. The observations of the other two fish were the same. In addition, all fish swimming in this study displayed the same swimming mode. Rome et al. (1993) reported that most of the power for swimming originates from the caudal muscle of scup (Stenotomus chrysops), whose swimming form seems to be subcarangiform or carangiform. Accordingly, I considered that zebrafish drive their caudal muscle during swimming. However, it is difficult to identify the specific part of the skeletal muscle in the caudal area that drives swimming the most in zebrafish because the bead of fast swimming fish was incompletely constant, and the part of the body that bends the most does not necessarily correspond to the skeletal muscle generating most of the propulsive force. Accordingly, I assigned the area of the axial section containing the posterior end of the anal fin junction to the mid-caudal half of the muscle mass and the area of the axial section containing the third abdominal vertebra to muscle studies isolated muscle and weighed each part. However, no study has isolated and weighed fish skeletal muscle because it has a different structure. Meulen et al. (2006) measured skeletal muscle cross-sectional area to study skeletal muscle hypertrophy during exercise in juvenile zebrafish. In their report, exercise quantity-dependent skeletal muscle hypertrophy was not shown clearly because growth rate in juvenile zebrafish is affected by skeletal muscle hypertrophy. Other zebrafish studies measured body weight (Palstra et al. 2010) or skeletal muscle fiber size (Palstra et al. 2014) to investigate the relationship between exercise and skeletal muscle mass. Accordingly, it is unclear how much exercise causes dose-dependent skeletal muscle hypertrophy. I determined the skeletal muscle area driving high-speed swimming. Each fish has a respective swimming mode, as the suffix "-form" (e.g., thunniform) refers to the type of movement and not to the body form. For example, the mode is classified based on the type of muscle used, such as caudal muscle or the trunk (e.g., anguilliform, subcarangiform, carangiform, thunniform, or ostraciiform) to the type using the dorsal, anal, or pectoral fin (e.g., amiiform, balistiform, tetraodontiform, gymnotiform, rajiform,

diodontiform, or labriform) for propulsive movement (Lindsey 1978). Although zebrafish are assumed to be the caudal type, such as anguilliform, subcarangiform, carangiform, thunniform, or ostraciiform, there was little detailed knowledge of zebrafish swimming mode until now. Therefore, I observed high-speed consecutive photographs of zebrafish swimming (Fig. 3) and the appearance (Fig. 2) of zebrafish with reference to Lindsey (1978), and considered that zebrafish are carangiforms because they have major characteristics of carangiforms, such as wavelength/body length >1.0; wavelength visible on body <0.5; amplitude/body length: undulations confined to posterior 1/3; body shape: mass concentrated anteriorly and peduncle quite narrow; and causal fin: posterior margin notched. The observations of the other two fish were the same. In addition, all fish swimming in this study displayed the same swimming mode. Rome et al. (1993) reported that most of the power for swimming originates from the caudal muscle of scup (Stenotomus chrysops), whose swimming form seems to be subcarangiform or carangiform. Accordingly, I considered that zebrafish drive their caudal muscle during swimming. However, it is difficult to identify the specific part of the skeletal muscle in the caudal area that drives swimming the most in zebrafish because the bead of fast swimming fish was incompletely constant, and the part of the body that bends the most does not necessarily correspond to the skeletal muscle generating most of the propulsive force. Accordingly, I assigned the area of the axial section containing the posterior end of the anal fin junction to the mid-caudal half of the muscle mass and the area of the axial section containing the third abdominal vertebra to the mid-cranial half of the muscle mass (Fig. 2) and compared these two parts using X-ray micro-CT. The results indicated that the mid-caudal half of the muscle hypertrophied in response to exercise training more than that of the mid-cranial half of the muscle (Fig. 4). In carp, which are closely related to zebrafish, the red muscle fibers run parallel to the body axis just under the skin, and the white muscle fibers (approximately 25% as long as red fibers) run in a helical

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fashion with respect to the spine (Rome 2002). These features suggest that the bending part directly contact the red muscle and that the part around the bending white muscle are the major drivers. Thus, hypertrophy of the caudal half of the muscle was considered reasonable. The morphometrics analysis of the mid-caudal half of muscle cross-sectional area revealed that the mid-caudal half of the muscle hypertrophied in response to exercise training (Table 2). The specific assignment of the axial section containing the posterior end of the anal fin junction as mid-caudal half of the muscle was considered to contribute largely to investigate muscle hypertrophy response to the quantity of exercise in detail.

My results demonstrate that forced exercise in zebrafish increased skeletal muscle mass dose dependently with the quantity of exercise in short-term. Several rodent studies have also shown a relationship between exercise quantity and skeletal muscle hypertrophy (Rodnick et al. 1989; Ishihara et al. 1998; Brown et al. 1992; McMahon et al. 2014). However, these studies were performed using a voluntary exercise wheel. In contrast, the zebrafish model has advantages of their innate behavior of swimming against water flow and the ability to undergo forced exercise without penalty or reward, as in many fish (McClelland 2012). A relatively heavily loaded exercise, which includes moving through the high density space of water, was forced uniformly on all exercised fish. In addition, zebrafish are diurnal, like humans. Zebrafish have a simple skeletal structure, and the caudal half of the zebrafish skeletal muscle generates most of the propulsive force. These advantages over rodents suggest that the zebrafish exercise model, in which exercise quantity can be accurately controlled and skeletal muscle can be easily analyzed with minimum influence (McClelland 2012), can be used, to accurately investigate the relationship between exercise quantity and skeletal muscle mass.

My results reveal skeletal muscle hypertrophy without an increase in body weight, although previous reports showed body growth with a suggestion of skeletal muscle hypertrophy by using

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larval or juvenile zebrafish (Meulen et al. 2006; Palstra et al. 2010). This study used mature adult zebrafish, which grow very little (Biga and Goetz 2006) or they were fed only enough to exercise. I am considering this topic for a future study under strict control of nutritional composition and intake.

The muscle fiber data (Fig. 6) show that hypertrophy of the muscle fibers increased muscle mass, as in mammals. In contrast, the red muscle hypertrophy was suggested after a minimal exercise (experiment 3) compared with that of white muscle (Table 2; Fig. 5). Red muscle is more active during sustainable swimming, but not during the escape response, in salmon or carp (Rome 2005), whose swimming mode is similar to that of zebrafish. The exercise pattern in this study was sustainable swimming; thus, zebrafish red muscle was considered activated and hypertrophied more than those of white muscle. Munoz et al. (1994) reported that voluntary wheel exercise by juvenile rats begins to increase (90% red muscle fibers) soleus muscle mass 1 week after starting the experiment and began to increase plantaris (90% white muscle fibers) muscle mass 2 weeks after starting the experiment. In my study, red muscle mass increased when exercise quantity was low, and white muscle mass increased as exercise quantity increased (Table 2; Fig. 5). Ishihara et al. (1991) reported that 6.5 weeks of voluntary wheel exercise by juvenile rats causes hypertrophy of the soleus and plantaris and increases fiber type composition and fiber area of fast-twitch oxidative glycolytic fibers, which are endurance-related white muscle fibers in the plantaris. The exercise conditions in my study increased endurance-related white muscle (data not shown), referred to as "pink muscle" in fish (Syme 2005). Thus, the exercise conditions in my study were equal to those of mammalian endurance exercise studies performed by Munoz et al. (1994) and Ishihara et al. (1991). The muscle fiber results indicated that the white muscle fiber size distribution did not change in experiment 2, even though the fibers became larger in experiments 1 and 3. Although the reason is unclear, it seems that insufficient caloric intake caused catabolism of white muscle, which was less activated than red

muscle. The increase in the feed intake ratio by exercise in experiment 2 (23%) was lower than that in experiment 1 (45%), despite that the exercise quantity for the first and second week duration was nearly equal between the two experiments.

PGC1α responds to exercise and growth of mitochondria, in part, through direct interactions with NRF1 in animals (Konopka and Harber 2014; Uguccioni et al. 2010; Lira et al. 2010; Lemoine et al. 2010b; Pilegaard et al. 2003), although Lemoine et al. (2010a) reported that the PGC1α gene expression level does not increase after acute exercise, but its putative target gene (the mitochondrial enzyme CS) increases 24 h after acute exercise. They discussed differences in PGC1α function between zebrafish and mammals. In contrast, acute exercise in my study increased PGC1α, NRF1, and CS gene expression levels. My exercise condition (42 cm/s) was harder than the condition in the study by Lemoine et al. (2010b) (9 cm/s). Accordingly, zebrafish PGC1α may be less sensitive than that of mammals and may respond to high-intensity exercise, as shown in my study. Additionally, the muscle-specific gene expression pattern, which is related to muscle catabolism and muscle cell proliferation and differentiation, suggested a muscle remodeling response to exercise. Expression of atrogin1 and MuRF1, which are muscle-specific ubiquitin ligases, increased after acute exercise. Increased atrogin1 and MuRF1 expression levels have also been reported in humans after acute exercise (Pasiakos et al. 2010). MyoD and myogenin gene expression, which facilitate muscle proliferation, differentiation, and regeneration, increased after acute exercise. These genes, particularly myogenin, increase in human skeletal muscle after acute resistance exercise (Yang et al. 2005). These genes could be responding to the amino acid supply for energy and muscle remodeling during and after exercise. Further study (e.g., using microarray, such as Palstra et al. 2014, or RNA-seq on red or white muscle) is needed to analyze the responses of skeletal muscle in detail. However, the gene expression patterns I observed suggest that exercise training affects muscle

remodeling and the properties of mitochondria.

My results demonstrate exercise quantity-dependent skeletal muscle hypertrophy for the first time in a forced exercise study. This is a significant outcome because the rodent model is not useful for investigating this relationship. The character of the skeletal muscle response in zebrafish, as shown in this study, may also contribute to investigations of motor neurons or neuromuscular junctions using the mutant, transgenic, or knockout zebrafish lines (Santoriello and Zon 2012; Daikoku et al. 2015; Kishi 2014).

Conclusions

The forced zebrafish exercise model allowed me to investigate the relationship between exercise quantity and skeletal muscle mass. I revealed that skeletal muscle hypertrophied with increasing exercise quantity, duration/day, and overall duration. Analyses of caudal half muscle, which was strongly activated during fast swimming, open the possibility of investigations on the effects of exercise on skeletal muscle. The results from this study demonstrate that zebrafish is a promising model to investigate the effect of exercise on skeletal muscle mass.

Figures
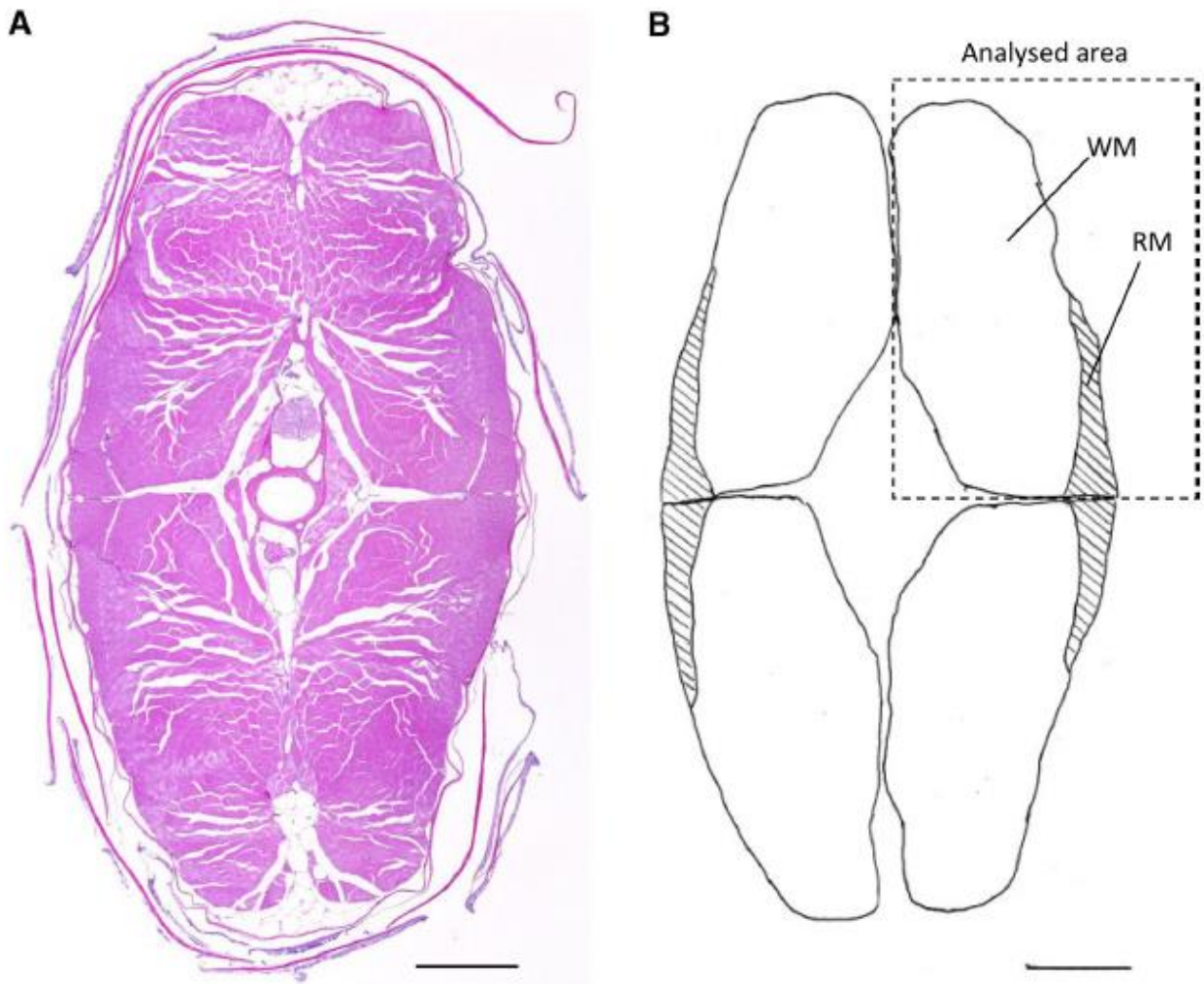
Fig. 1   Zebrafish cross-sectional muscle diagram.
A Hematoxylin–eosin (HE) stained cross-section of the caudal muscle (Fig. 2, line B). **B** Diagram of the cross-section. RM, red muscle fibers; WM white muscle fibers. All muscle area and muscle fiber size measurements were performed in the "analyzed area" within the dotted line square. Scale bars in **A** and **B** 500 μm

Fig. 2　Cross-sectional position for X-ray micro computed tomography (CT) and histological analysis.
Cross-section at lines A midcranial half muscle and B mid-caudal half muscle in zebrafish were measured and analyzed as cranial skeletal muscle and caudal skeletal muscle, respectively, by micro-CT. Histological analysis was performed at line B

Table 1 Changes in water flow velocity

Experiment no.	Water flow velocity (exercise quantity per week)			
	1st week	2nd week	3rd week	4th week
Experiment 1 (6 h/day for 4 weeks)	43 cm/s (30 h)	48 cm/s (30 h)	52 cm/s (30 h)	52 cm/s (30 h)
Experiment 2 (6 h/day for 2 weeks)	43 cm/s (30 h)	48 cm/s (30 h)	–	–
Experiment 3 (3 h/day for 2 weeks)	43 cm/s (15 h)	48 cm/s (15 h)	–	–

Water flow velocity (swimming speed) was equal to swimming speed and was adjusted for the maximum velocity at which the fish could continue swimming and was readjusted once/week. Exercise quantity per week is shown (in parentheses)

Table 2 Effect of exercise on body weight and skeletal muscle cross-sectional area

Experiment no.	Group	n	Initial	Endpoint		
			Body weight (mg)	Muscle cross-sectional area (mm^2)		
				Red muscle	White muscle	Total muscle
Experiment 1 (6 h/day for 4 weeks)	Non-exercised	12	846.2 ± 15.4 858.8 ± 24.2	0.09023 ± 0.00404	0.97129 ± 0.03588	1.06152 ± 0.03799
	Exercised	12	848.8 ± 25.4 814.0 ± 37.8	0.12769 ± 0.00563***	1.11275 ± 0.04271*	1.24044 ± 0.04730**
Experiment 2 (6 h/day for 2 weeks)	Non-exercised	8	728.1 ± 21.0 755.6 ± 18.9	0.09400 ± 0.00383	1.08450 ± 0.03463	1.17850 ± 0.03771
	Exercised	8	731.0 ± 19.0 717.9 ± 16.0	0.12099 ± 0.00327***	1.13671 ± 0.03053	1.25770 ± 0.03033
Experiment 3 (3 h/day for 2 weeks)	Non-exercised	8	757.5 ± 21.4 772.6 ± 20.1	0.08760 ± 0.00436	0.93685 ± 0.02908	1.02445 ± 0.03330
	Exercised	8	760.8 ± 22.2 762.4 ± 21.7	0.10100 ± 0.00408*	0.91942 ± 0.04480	1.02043 ± 0.04713

Significant values between non-exercised and exercised groups for each experiment: *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$
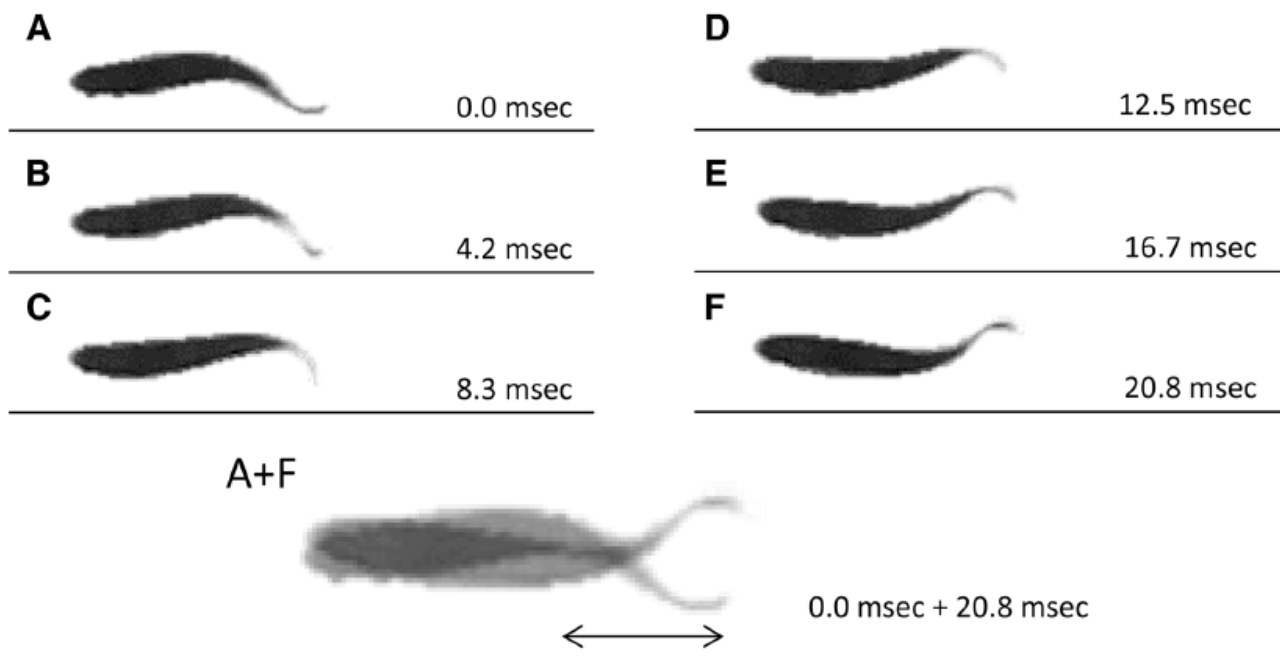
Fig. 3 Consecutive high-speed photographs of zebrafish during fast swimming.
All photographs were taken with a high-speed digital camera (240 frames/s) from the dorsal side. a–f Consecutive photographs during half of a tail beat cycle (20.8 ms). g A + F overlay. The body part indicated by the two-headed arrow contracts and bends the body and generates most of the propulsive force
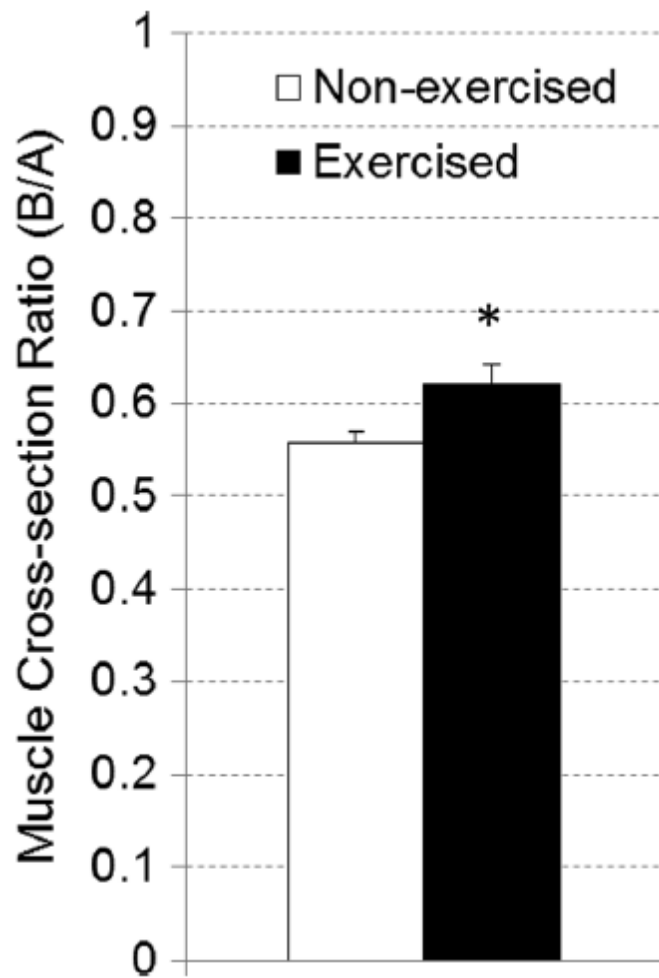
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Fig. 4 Muscle hypertrophy in zebrafish after swimming exercise.
The ratio of caudal skeletal muscle cross-sectional area (Fig. 2, line B) to cranial skeletal muscle cross-sectional area (Fig. 2, line A) was analyzed by X-ray micro- computed tomography. Values are means ± standard errors. Significant values between non-exercised and exercised groups: $*p < 0.05$
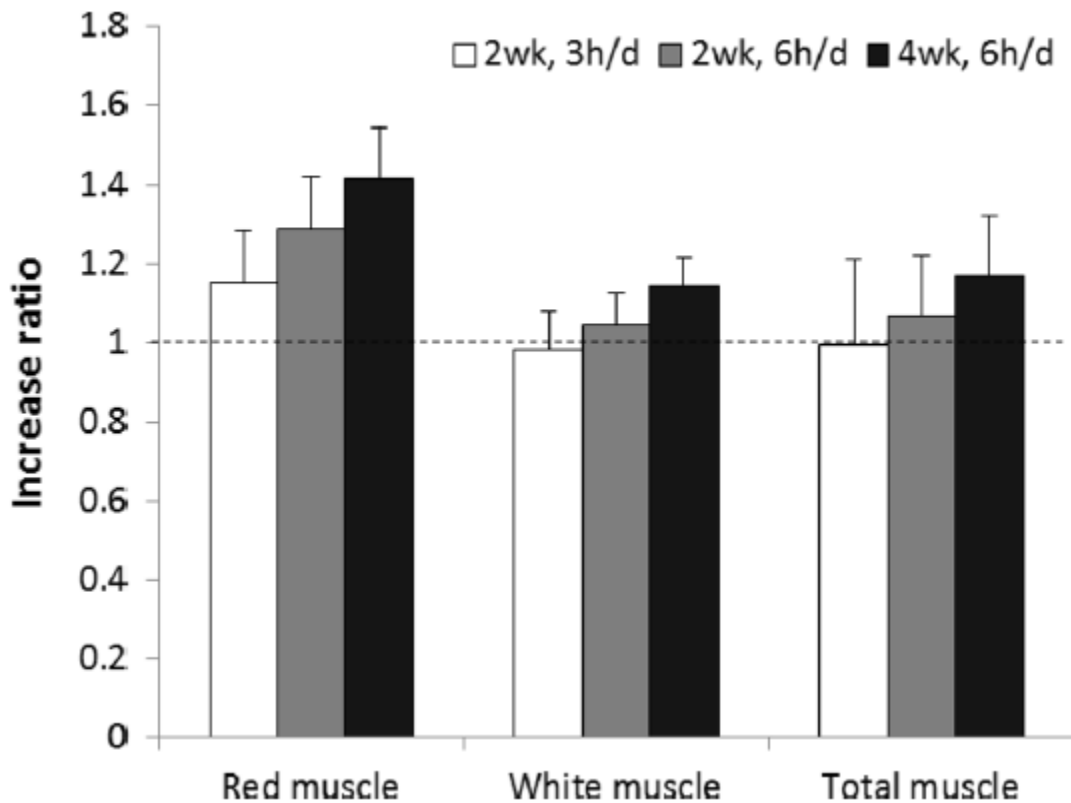
Fig. 5   Effects of changing exercise quantity on increases in the skeletal muscle ratio.
The skeletal muscle mass ratio of each muscle type increased with each increase in exercise quantity; 2 weeks, 3 h/day in experiment 3; 2 weeks, 6 h/day in experiment 2; and 4 weeks 6 h/day in experiment 1. The ratio values are means ± standard deviations. Increasing trend in the muscle ratio with the increase in exercise quantity was detected by the Jonckheere–Terpstra test for a trend in red muscle ($p < 0.01$), white muscle ($p < 0.01$), and total muscle ($p < 0.01$)
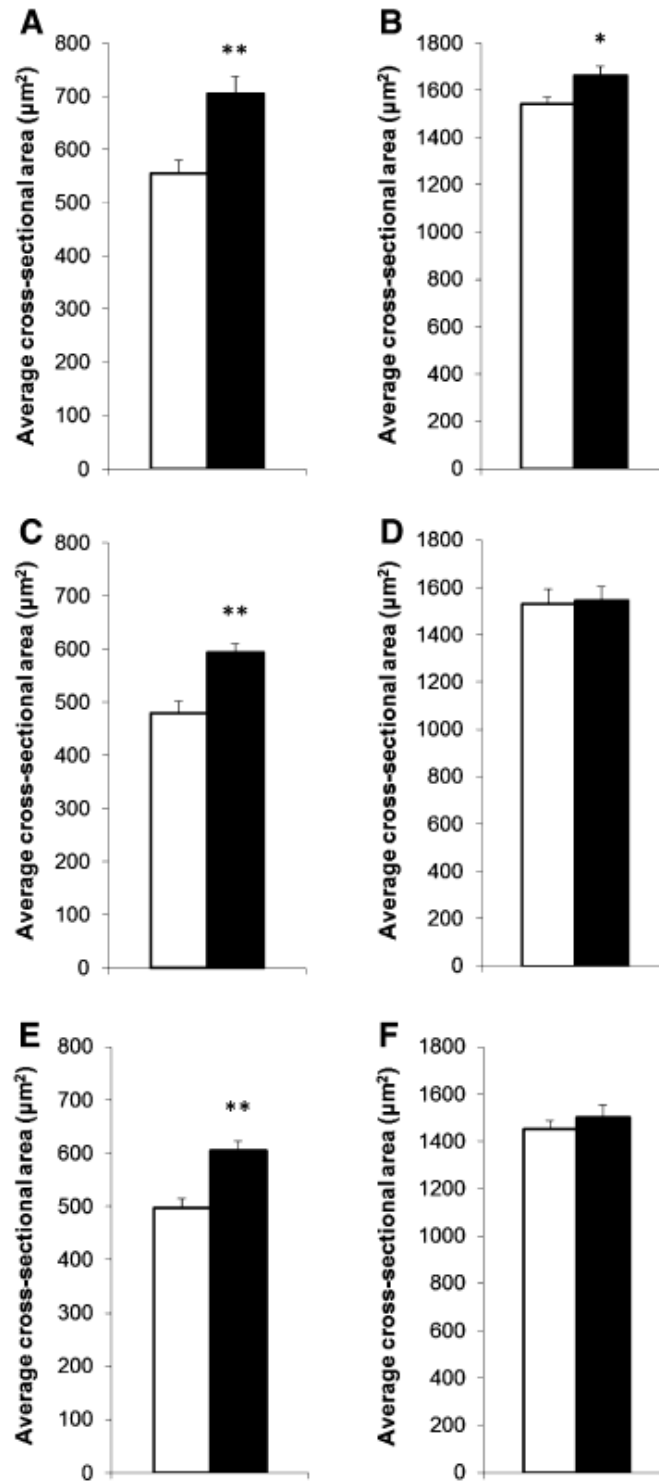
Fig. 6 Morphometric analysis of mean muscle fiber area in zebrafish after swimming exercise.
A Mean, red muscle fiber cross-sectional area in the non-exercised and exercised groups in experiment 1. **B** Mean, white muscle fiber cross-sectional area in experiment 1. **C** Mean, red muscle fiber cross-sectional area in experiment 2. **D** Mean, white muscle fiber cross-sectional area in experiment 2. **E** Mean, red muscle fiber cross-sectional area in experiment 3. **F** Mean, white muscle fiber cross-sectional area (right side) in experiment 3. All muscle fiber cross-sectional values are means $\pm$ standard errors. Significant values between non-exercised and exercised groups: **p < 0.01,

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and *p < 0.05
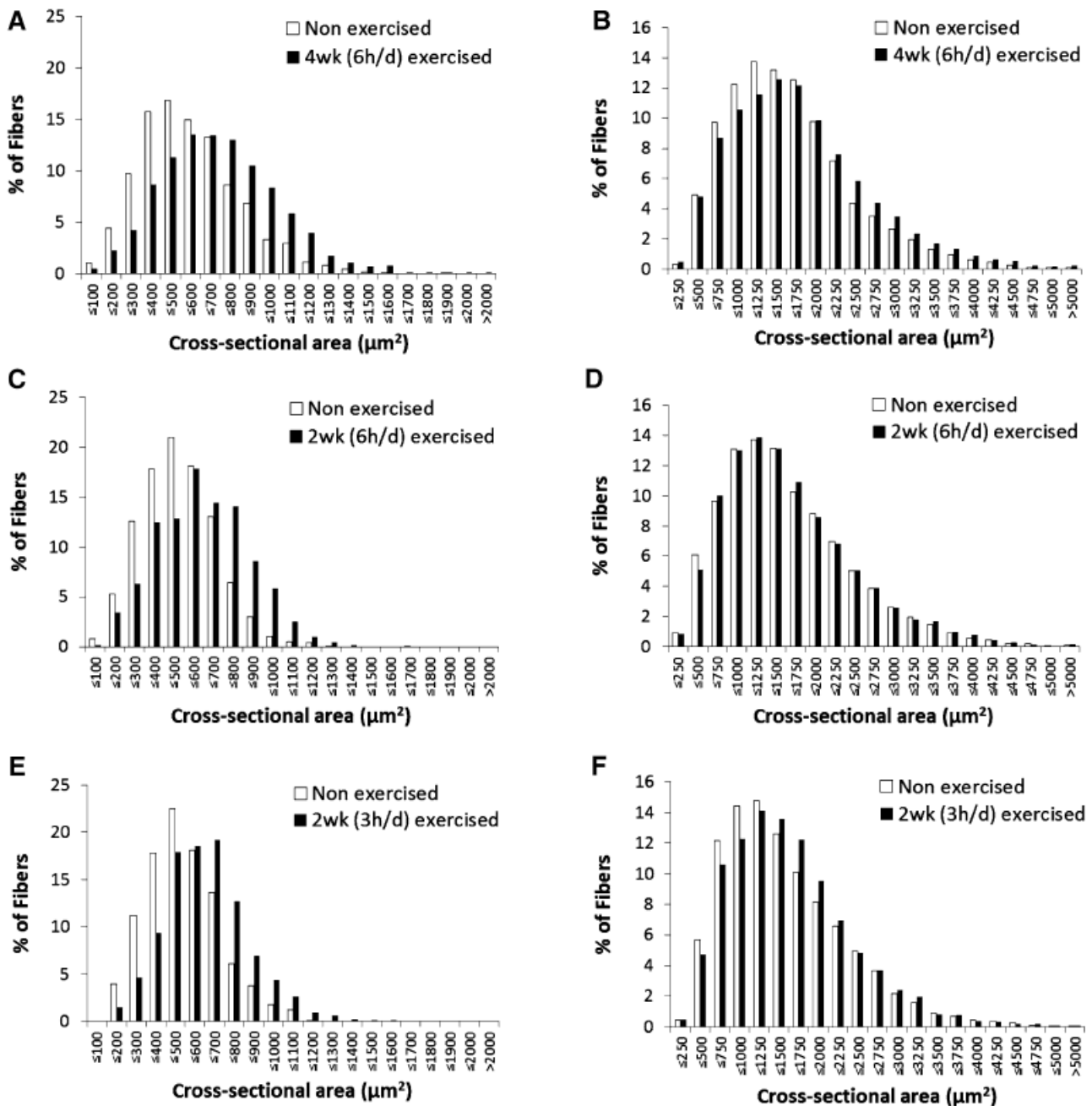
Fig. 7 Histogram of the morphometric analysis results of zebrafish muscle fibers after swimming exercise.

A Histogram of red muscle fiber cross-sectional area in the non-exercised and exercised groups for experiment 1. **B** Histogram of white muscle fiber cross-sectional area in experiment 1. **C** Histogram of red muscle fiber cross-sectional area in experiment 2. **D** Histogram of white muscle fiber cross-sectional area in experiment 2. **E** Histogram of red muscle fiber crosssectional area in experiment 3. **F** Histogram of white muscle fiber cross-sectional area in experiment 3. All muscle fiber cross-sectional values are means ± standard errors. Transit distribution of muscle fibers, except white muscle fibers in experiment 2 (d), to a large size was detected by the Mann–Whitney U test. Red muscle fiber (p < 0.001) and white muscle fiber (p < 0.001) in experiment 1; red muscle fiber (p < 0.01) in experiment 2; red muscle fiber (p < 0.001) and white muscle fiber (p < 0.001) in
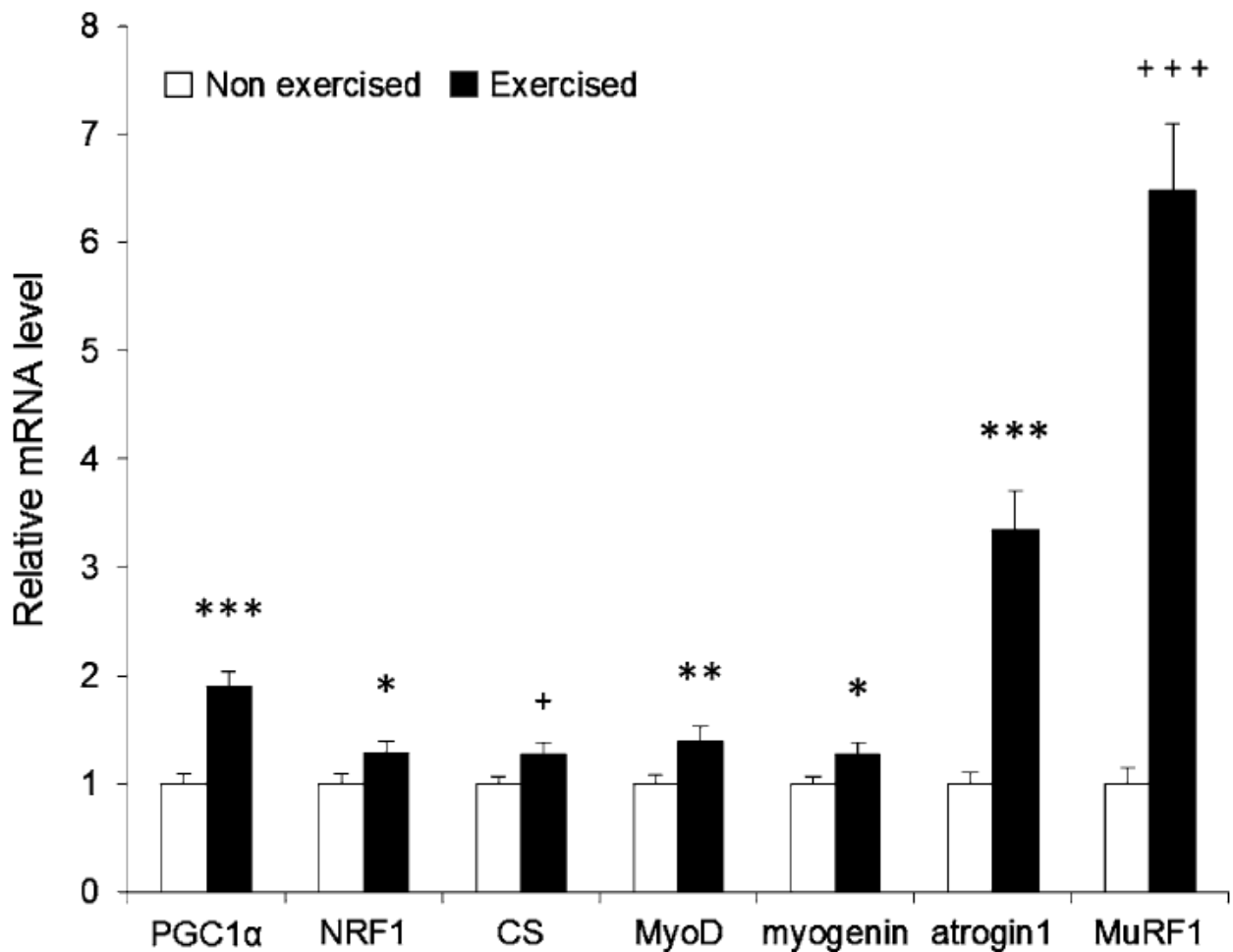
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experiment 3

Fig. 8   Effects of a single exercise on skeletal muscle gene expression.
Peroxisome proliferator-activated receptor gamma and coactivator 1 alpha (PGC1α), nuclear respiratory factor 1 (NRF1), citrate synthase (CS), myogenic differentiation 1 (MyoD), myogenin, atrogin1, and MuRF1 mRNA expression levels in the caudal skeletal muscle half after acute exercise in experiment 4. Expression of each mRNA was normalized against corresponding expression in the non-exercised group. Student's t test was used for the PGC1α, NRF1, MyoD, myogenin, and atrogin1 expression levels by because the data were normal. The Mann–Whitney U test was used to test CS and MuRF1 expression levels because the data were not normal. Values are means ± standard errors. Significant values between non-exercised and exercised groups: ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ by Student's t test; +++$p < 0.001$, +$p < 0.05$ by Mann–Whitney U test

Part II

Green Tea Extract Suppresses Adiposity and Affects the Expression of Lipid Metabolism Genes in Diet-Induced Obese Zebrafish

Abstract

Visceral fat accumulation is one of the most important predictors of mortality in obese populations. Administration of green tea extract (GTE) can reduce body fat and reduce the risk of obesity-related diseases in mammals. In this study, I investigated the effects and mechanisms of GTE on adiposity in diet-induced obese (DIO) zebrafish.

Zebrafish at 3.5 to 4.5 months post-fertilization were allocated to four groups: non-DIO, DIO, DIO + 0.0025%GTE, and DIO + 0.0050%GTE. The non-DIO group was fed freshly hatched Artemia once daily (5 mg cysts/fish daily) for 40 days. Zebrafish in the three DIO groups were fed freshly hatched Artemia three times daily (60 mg cysts/fish daily). Zebrafish in the DIO + 0.0025%GTE and DIO + 0.0050%GTE groups were exposed to GTE after the start of feeding three times daily for 40 days.

Three-dimensional microcomputed tomography analysis showed that GTE exposure significantly decreased the volume of visceral but not subcutaneous fat tissue in DIO zebrafish. GTE exposure increased hepatic expression of the lipid catabolism genes ACOX1 (acyl-coenzyme A oxidase 1, palmitoyl), ACADM (acyl-coenzyme A dehydrogenase, c-4 to c-12 straight chain), and PPARA (peroxisome proliferator-activated receptor alpha). GTE exposure also significantly decreased the visceral fat expression of SOCS3 (suppressor of cytokine signaling 3b) which inhibits leptin signaling.

The present results are consistent with those seen in mammals treated with GTE, supporting the validity of studying the effects of GTE in DIO zebrafish. My results suggest that GTE exerts beneficial effects on adiposity, possibly by altering the expression of lipid catabolism genes and SOCS3.

Introduction

According to 2005 World Health Organization estimates, approximately 1.6 billion adults worldwide were overweight (body mass index [BMI] ≥ 25 kg/m^2) and at least 400 million were obese (BMI $\geq$ 30 kg/m^2), and these numbers are expected to reach 2.3 billion and 700 million, respectively, by 2015 (Malik et al. 2010). It is widely accepted that obesity results from a positive energy balance (Spiegelman et al. 2001). In general, obesity can be classified into visceral and subcutaneous types according to the distribution of fat. Visceral obesity accompanies or precedes components of the metabolic syndrome, such as hyperinsulinemia and insulin resistance (Ibrahim 2010). Consequently, visceral fat accumulation is an independent predictor of mortality in men (Kuk et al. 2006).

Green tea, one of the most popular beverages in Asian countries, contains numerous polyphenols known as catechins, particularly epigallocatechin gallate, epicatechin gallate, and gallocatechin gallate. Tea and tea components have many beneficial properties, including antioxidant (Rice-Evans et al. 1996), anticancer (Shankar et al. 2007; Zaveri 2006)), antidiabetic (Matsumoto et al. 1993), and anti-atherogenic (Miura et al. 2001) activities. Moreover, intake of tea catechins inhibits DIO in mice (Klaus et al. 2005; Murase et al. 2002) and reduces body weight and body fat in humans (Kozuma et al. 2005; Nagao et al. 2005, 2007; Takase et al. 2008; Takeshita et l. 2008; Zhang et al. 2012). These anti-obesity effects of tea catechins seem to involve stimulation of fat oxidation (Klaus et al. 2005; Dulloo et al. 1999; Shimotoyodome et al. 2005), modulation of adipogenesis (Furuyashiki et al. 2004), decreased fat synthesis (Shankar et al. 2007; Furuyashiki et al. 2004; Ikeda et al. 2005; Watanabe et al. 1998), and inhibition of digestive enzyme activity and nutrient absorption (Matsumoto et al. 1993).

Zebrafish (*Danio rerio*) are vertebrates, and their organs and tissues show similarities to those of humans in terms of their structure and function. Consequently, zebrafish are increasingly

being used as models of human diseases (Kozuma et al. 2005; Best et al. 2008; Kishi et al. 2009; Lieschke et al. 2007) because of their ease of genetic manipulation and their economic potential for use in breeding and testing. In addition, lipid metabolism in zebrafish is very similar to that in humans in terms of intestinal absorption with the aid of bile produced in the liver (James et al. 1987), transport of fat and cholesterol by lipoproteins (Hölttä-Vuori et al. 2010), β-oxidation (Morais et al. 2007), and storage as triacylglycerols in visceral, subcutaneous, and intramuscular adipocyte depots (James et al. 1987; Song et al. 2007). Because of these similarities and advantages, zebrafish are used in lipid metabolism research as a model for lipid-related diseases, including atherosclerosis induced by high-cholesterol diets (Stoletov et al. 2009) and obesity induced by overexpression of the endogenous melanocortin antagonist agouti-related protein (AgRP) (Song et al. 2007). Moreover, Oka et al. demonstrated the usefulness of a zebrafish model of DIO that shares common pathophysiological pathways with mammalian obesity (Oka et al. 2010). DIO zebrafish have also been used to validate the antiobesity effects of natural products (Tainaka et al. 2011).

Here, I investigated the effects of GTE on body weight, visceral and subcutaneous fat accumulation, and the expression of lipid metabolism genes in the liver and visceral fat in DIO zebrafish.

Materials and methods

Ethical approval

This study conformed to the ethical guidelines established by the Institutional Animal Care and Use Committee of Mie University.

GTE

GTE was prepared and analyzed as previously described (Murase et al. 2006). In brief, green tea leaves (*Camellia sinensis*) were soaked in hot water and the resulting extract was reduced to a powder by spray drying. The extract was then dissolved in hot water and mixed with an equal volume of chloroform. The aqueous phase was recovered with three volumes of ethanol and the extract was freeze-dried after removing the solvent. The composition of catechins was measured by high-performance liquid chromatography. The total catechin content in the GTE was 79.6%−81.8% (w/w, the sum of all catechins); this comprised epigallocatechin gallate (43.6%−44.4%), epigallocatechin (20.4%−20.7%), epicatechin gallate (12.0%−12.3%), epicatechin (8.1%−8.3%), gallocatechin (6.9%−7.0%), gallocatechin gallate (4.4%), and others (3.7%−3.8%). The caffeine content was 0%−0.1%.

Animals

Male and female zebrafish (AB strain, the Zebrafish International Resource Center, Eugene, OR, USA) were kept at approximately 28°C under a 14-h light and 10-h dark cycle. Water conditions of environmental quality were maintained according to The Zebrafish Book (Westerfield 2000).

Experimental design

Experiment 1

Male and female zebrafish at 3.5–4.5 months postfertilization were allocated to four groups (non-DIO, DIO, DIO + 0.0025%GTE, and DIO + 0.0050%GTE) with five fish per 1.7-L tank. Zebrafish in the non-DIO (control group) were fed freshly hatched Artemia for 120 min once a day (5 mg cysts/fish daily) for 40 days. Zebrafish in the three DIO groups were fed freshly hatched Artemia for 120 min three times daily (60 mg cysts/fish daily). Zebrafish in the DIO + 0.0025%GTE and DIO + 0.0050%GTE groups were exposed to GTE for 105 min, starting 15 min after the start of feeding (total exposure, 315 min/day), three times daily for 40 days. During feeding, the tank water flow was stopped; the water in each tank was replaced with fresh water at the end of feeding. Body weight was measured on days 0, 14, 20, 27, 34, and 40. Body fat volume was measured using three-dimensional microcomputed tomography (3D micro-CT) following euthanasia on the final day of the study.

Experiment 2

Female zebrafish were allocated to three groups (non- DIO, DIO, and DIO + 0.0050%GTE) with five fish per 1.7-L tank. All groups were fed as described in Experiment 1 for 21 days. After feeding on the final day, the zebrafish were euthanized, immediately transferred into tubes containing 8 mL of RNAlater (Qiagen, Valencia, CA, USA), and stored at 4°C until gene expression analysis.

CT measurement of body fat volume

Zebrafish were fixed in a stretched position on a sample holder. The 3D micro-CT scan was performed with an in vivo System R_mCT 3D micro-CT scanner (Rigaku Corporation, Tokyo, Japan). The following settings were used: voltage, 90 kV; current, 100 µA; magnification, ×4; slice

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thickness (scanning width), 50 μm; and exposure time, 2 min. Images were reconstructed and viewed using i-View type R software (J. Morita Mfg., Kyoto, Japan). The CT images were visualized and analyzed using CTAtlas Metabolic Analysis Ver. 2.03 software (Rigaku Corporation). The Hounsfield unit (HU) value of fat tissue was adjusted to between -350.0 and -145.0 in accordance with the manufacturer's instructions. Measurement of body fat volume was limited to the abdominal cavity, and the initial point of the abdominal cavity was set at the cleithrum (Fig. 9A). Body fat was then divided into visceral fat and subcutaneous fat along the ribs (Fig. 9B).

RNA extraction and quantitative real-time PCR

The liver and visceral fat were dissected from the stored fish samples prepared in Experiment 2 and were subjected to RNA extraction. Total RNA was extracted from the livers of fish in all three groups (non-DIO, DIO, and DIO + 0.0050%GTE) using Isogen (Nippongene, Tokyo, Japan) with combination with cleanup protocol of RNeasy mini kit (Qiagen K. K., Tokyo, Japan). cDNA was synthesized using random primers and SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). Total RNA was extracted from the visceral fat of fish in the DIO and DIO + 0.0050%GTE groups using an RNeasy Lipid tissue mini kit (Qiagen) and qualified using an Agilent Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA). Because of poor RNA quality, the samples for two of five fish in the DIO group were excluded. cDNA was synthesized using a High Capacity RNA-to cDNA Kit (Applied Biosystems, Foster City, CA, USA). Total RNA could not be extracted from the visceral fat of fish in the non-DIO group because the amount of visceral fat was too small.

Quantitative real-time PCR was performed on cDNA samples using a TaqMan Fast Universal PCR Master Mix (Applied Biosystems) or Fast SYBR Green Master Mix (Applied Biosystems) and an ABI Prism 7500 Fast Real-Time PCR System (Applied Biosystems) in

accordance with the manufacturer's instructions. The TaqMan gene expression assays were as follows: PPIA (peptidylprolyl isomerase Aa; Dr03152038_m1), ACOX1 (acyl-coenzyme A oxidase 1, palmitoyl; Dr03147239_m1), ACADM (Dr03120754_m1), PPARA (peroxisome proliferator-activated receptor alpha b; Dr03149883_m1), and SOCS3 (suppressor of cytokine signaling 3b; Dr03203997_s1). The primer sets for SYBR Green were as follows: ACC (acetyl-CoA carboxylase gene, XM_678989, forward primer (5′–3′): ATCATCCCACCCAAACAGAC; reverse primer (3′–5′): CCCATCACAGAAGGTGGAAC) and FASN (fatty acid synthase gene, XM_682295, forward primer (5′–3′): ATCTGTTCCTGTTCGATGGC, reverse primer (3′–5′): AGCATATCTCGGCTGACGTT). The baseline and threshold were set manually in accordance with the manufacturer's instructions. The relative mRNA expression levels were determined using PPIA as an endogenous standard.

Feeding volume assay

The feeding volume assay was conducted as previously described (Tainaka et al. 2011), with minor modifications, on day 39 of Experiment 1. Briefly, hatched *Artemia* (5 or 60 mg cysts/fish/day) were fed to the zebrafish in a 1.7-L tank as described above. For a blank control, *Artemia* were placed in a 1.7-L tank without zebrafish (the tank contained water alone). After 90 min, the number of *Artemia* not eaten by the zebrafish were counted three times and subtracted from the number in the blank tank to determine the feeding volume in each tank.

Statistical analysis

All values are presented as means ± standard error of the mean. Statistical analysis was conducted using analysis of variance followed by Fisher's partial least-squares difference multiple

comparison test. Analyses were conducted using STATVIEW for Windows version 5.0 (SAS Institute

Inc., Cary, NC, USA). Values of P < 0.05 were considered statistically significant.

Results

Food intake

Visual observation revealed no marked abnormalities or major differences in feeding behavior between the three DIO groups (i.e., DIO, DIO + 0.0025%GTE, and DIO+ 0.0050%GTE). The ratio of Artemia consumed to the amount of Artemia provided (consumption ratio) was estimated in the feeding volume assay. In male zebrafish, the consumption ratio in the DIO+ 0.0025%GTE (40%) and DIO + 0.0050%GTE (37%) did not decrease compared with that in the DIO group (24%). And also in female, the consumption ratio in the DIO+ 0.0025%GTE (33%) and DIO+ 0.0050%GTE (38%) did not decrease compared with that in the DIO group (33%).

Body weight

Significant increases in body weight were observed within 14 days of the start of the experiment in male and female zebrafish in the DIO group compared with those in the non-DIO group (Fig. 10). This trend was maintained throughout the 40-day study. From day 14 onward, exposure to 0.0050% GTE significantly reduced the diet-induced body weight gain in females (P < 0.05 or P < 0.01 compared with the DIO group). By contrast, there were no significant reductions in diet-induced body weight gain in males exposed to either dose of GTE, or in females exposed to 0.0025% GTE.

Body fat volume

To quantify body fat volume in DIO zebrafish, I performed 3D micro-CT analysis. This allowed us to quantify the total body fat volume and to separately quantify the visceral and subcutaneous fat volumes. Fig. 11 compares the body fat volumes between each experimental group.

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Total body fat, visceral fat, and subcutaneous fat volumes in both sexes in the DIO group were significantly greater than those in the non-DIO group. Total body fat and visceral fat volumes in male DIO zebrafish were significantly reduced by exposure to 0.0050% GTE, and those in female DIO zebrafish were significantly reduced by both doses of GTE. However, in both sexes, there were no significant differences in subcutaneous fat volume between the DIO group and either GTE-treated group.

Liver and visceral fat mRNA expression in females

I analyzed the mRNA expression levels of lipid metabolism genes in the liver and visceral fat tissue of female DIO zebrafish because the suppressive effects of GTE on body fat accumulation were greater in females than in males.

In the liver on day 21 of the experiment, the mRNA expression levels of the mRNAs encoding ACOX1 (a peroxisomal β-oxidation enzyme), ACADM (a mitochondrial β-oxidation enzyme), and PPARA (a nuclear receptor protein that regulates β-oxidation) were significantly upregulated in the DIO + 0.0050%GTE group compared with the DIO group (Fig. 12. By contrast, there was no significant difference in the mRNA expression of SOCS3 (a protein that suppresses leptin signaling).

In the visceral fat on day 21 of the experiment, the mRNA expression levels of ACOX1, ACADM, and PPARA in the DIO+ 0.0050%GTE group were not significantly differ from those in the DIO group. In addition, there were no significant differences in the mRNA expression levels of FASN (fatty acid synthase) or ACC (acetyl-CoA carboxylase), which play important roles in lipogenesis. On the other hand, the mRNA expression of SOCS3 was significantly lower in the DIO+ 0.0050%GTE group than in the DIO group (Fig. 13).

Discussion

In this study, I demonstrated that GTE significantly suppressed the accumulation of visceral fat, but not subcutaneous, in a concentration-dependent manner in zebrafish. This effect was accompanied by increase hepatic expression of lipid catabolism genes. These results are very consistent with the findings of previous studies in humans (Kozuma et al. 2005; Nagao et al. 2007) and rodents (Klaus et al. 2005; Murase et al. 2002; Nagao et al. 2005; Chen et al. 2009; Lee et al. 2009; Sae-Tan et al. 2011). Although it is unclear whether the functions of visceral and subcutaneous fat in zebrafish are homologous to those in mammals, my findings indicate that body fat distribution is present in zebrafish as well as in humans and animals and that the site-specific effects of GTE are similar to those in mammals.

The mechanisms underlying the suppressive effects of GTE on body fat accumulation have been investigated in rodent models. Some studies have shown that upregulated PPARs and many lipid-metabolizing enzymes stimulate fat oxidation in the liver (Murase et al. 2002; Chen et al. 2009; Lee 2009). The hepatic gene expression pattern observed in DIO zebrafish in my study support these earlier findings. Although it was recently reported that the expression of lipogenic genes, such as FASN, is downregulated in visceral fat tissue in obese mice (Park et al. 2011), similar changes were not observed in my study. I assume that the reason for this discrepancy is the difference in obesity models used. In particular, Park et al. used obese mutant mice with increased FASN gene expression in adipose tissue (Park et al. 2011), whereas I used a wild-type strain of fish. In support of the hypothesis that the activities of FASN differs between models of obesity, Letexier et al. reported that adipose tissue FASN expression is not affected by a high-fat diet in wild-type rodents and is reduced in obese humans (Letexier et al. 2003). Their results help support the notion that adipose tissue gene expression profiles differ among species, and the lack of consistency between my data and those of

Park et al.(2009). Nevertheless, my present results suggest that the mechanism by which GTE suppresses body fat accumulation, at least in the liver, in zebrafish is similar to that in rodents.

I also found that GTE significantly decreased the expression of SOCS3 in the visceral fat of DIO zebrafish. SOCS3 is a negative regulator of leptin signaling and was recently proposed as an important therapeutic target for obesity (Howard et al. 2004). SOCS3 production in fat is associated with obesity in humans and rodents as SOCS3 gene expression is increased in subcutaneous fat of obese patients (Rieusset et al. 2004), and its protein and gene expression levels are increased in epididymal fat of DIO rodents (Kanatani et al. 2007; Gu et al. 2009). I found that GTE suppressed adipose tissue SOCS3 expression in zebrafish, which was accompanied by reduced body fat accumulation. To my knowledge, these results suggest for the first time that SOCS3 expression is correlated with body fat volume in zebrafish and is involved in the regulation of body fat volume by GTE.

Another valuable outcome of this study is that 3D micro-CT analysis can be used to analyze the effect of compounds on adiposity in DIO zebrafish. In humans, visceral fat accumulation, unlike subcutaneous fat accumulation, is strongly associated with metabolic and vascular risks, particularly diabetes, hypertension, and stroke, and thus increases the risk of mortality (Ibrahim et al. 2010). CT is widely used to measure body fat volume in humans (Cnop et al. 2003; Matsuzawa et al. 2002) and in rodents (Judex et al. 2010; Luu et al. 2009). Although CT is used to evaluate fillet composition in teleosts, such as salmon and carp (Folkestad et al. 2008; Nanton et al. 2007; Romvári et al. 2002), to my knowledge, no reports have used CT to measure body fat volume or visceral/subcutaneous fat volume in small teleosts. My data raise the possibility that micro-CT could be used to measure body fat volume in such fish. I believe this technique will play an important role in future studies of lipid metabolism and obesity in zebrafish.

Conclusions

I showed that GTE significantly inhibits weight gain and body fat accumulation, and alters the expression of hepatic lipid catabolism genes in DIO zebrafish. My results suggest that GTE exerts beneficial effects on adiposity, possibly by altering the expression of lipid catabolism genes and SOCS3.
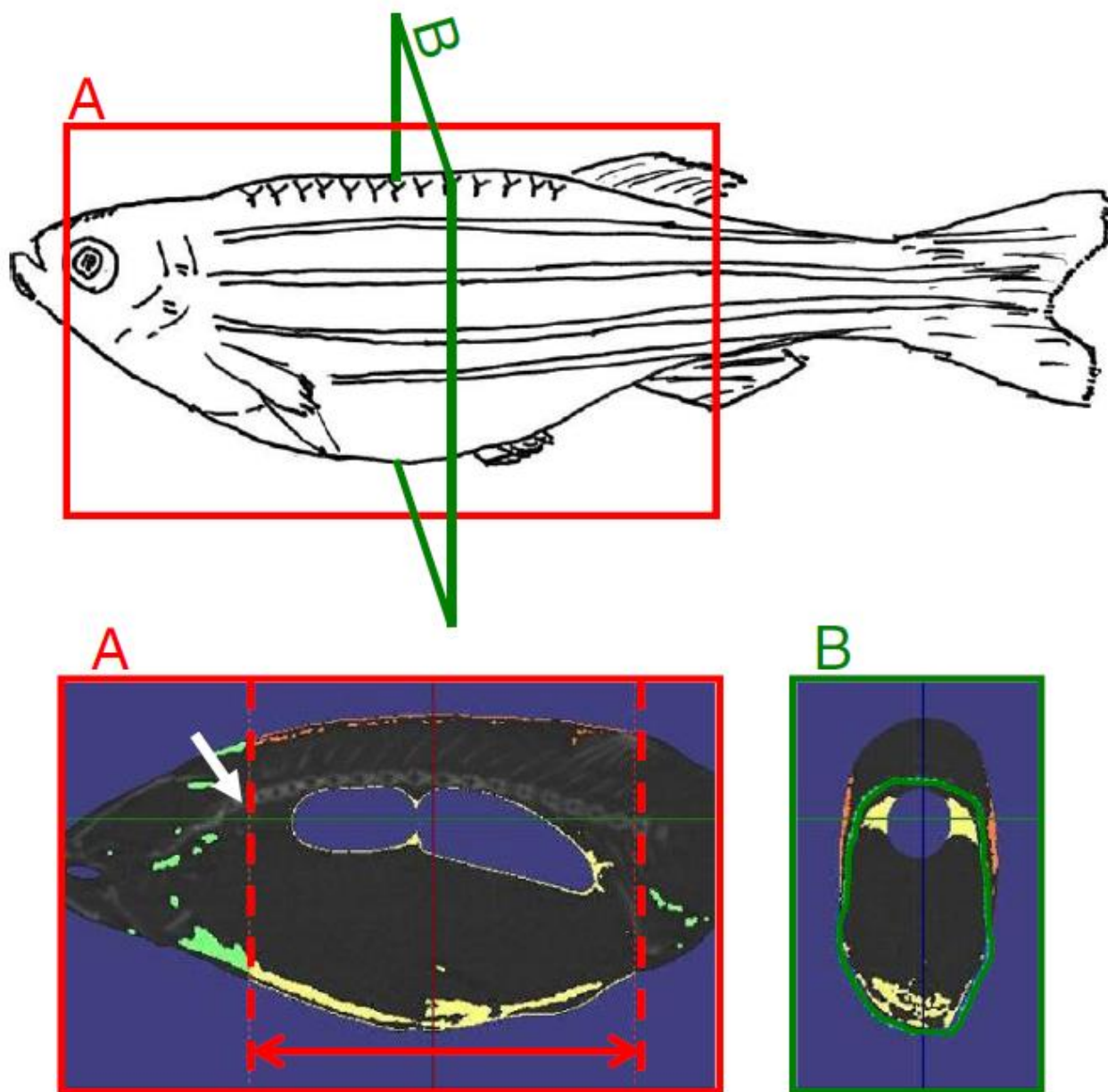
Figures

Fig. 9   Cross-sectional images taken by three-dimensional micro-computed tomography.

The diagram of the zebrafish shows where the two cross-sectional images A and B were taken. (A) The red two-headed arrow shows the area where body fat volume was measured (yellow). (B) The green line shows the boundary between the visceral fat and the subcutaneous fat (orange). The light green area indicates body fat outside the measuring area. The white arrow indicates the cleithrum.
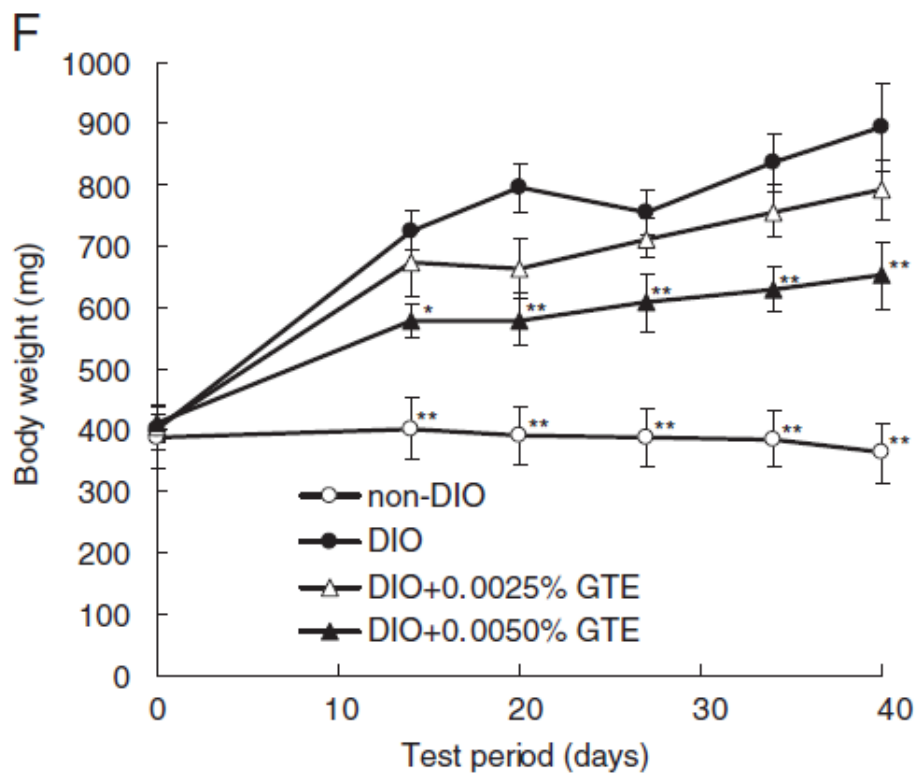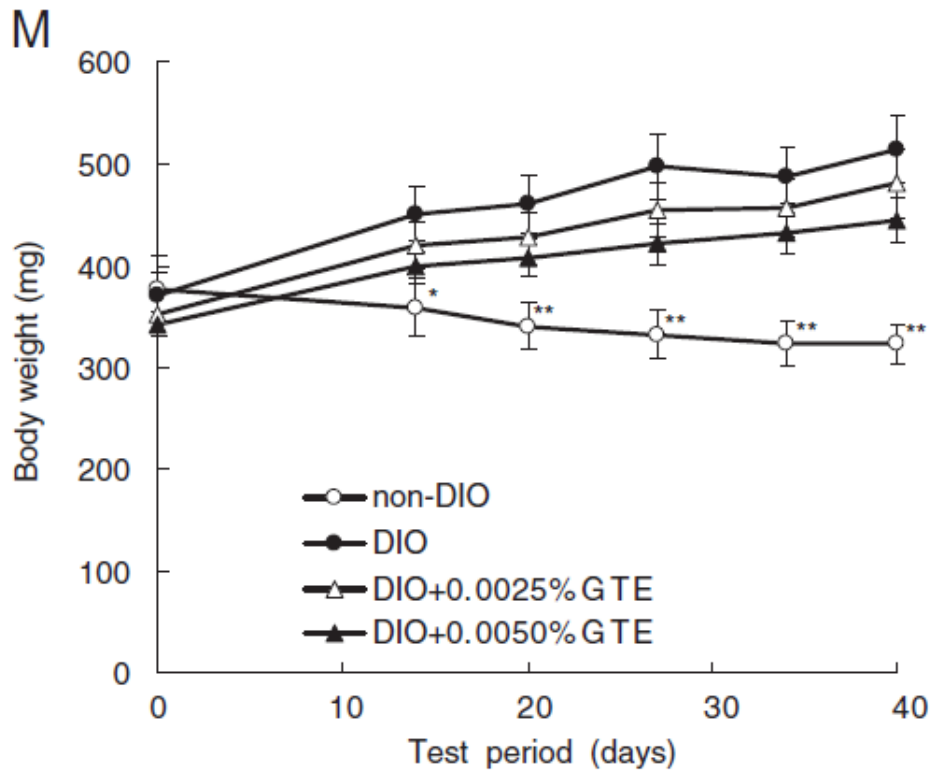
Fig. 10   Effects of green tea extract (GTE) on body weight.

Changes in body weight in male (M) and female (F) zebrafish. Values are means ± SE. **$P < 0.01$

and *$P < 0.05$ vs. the diet-induced obese (DIO) group.
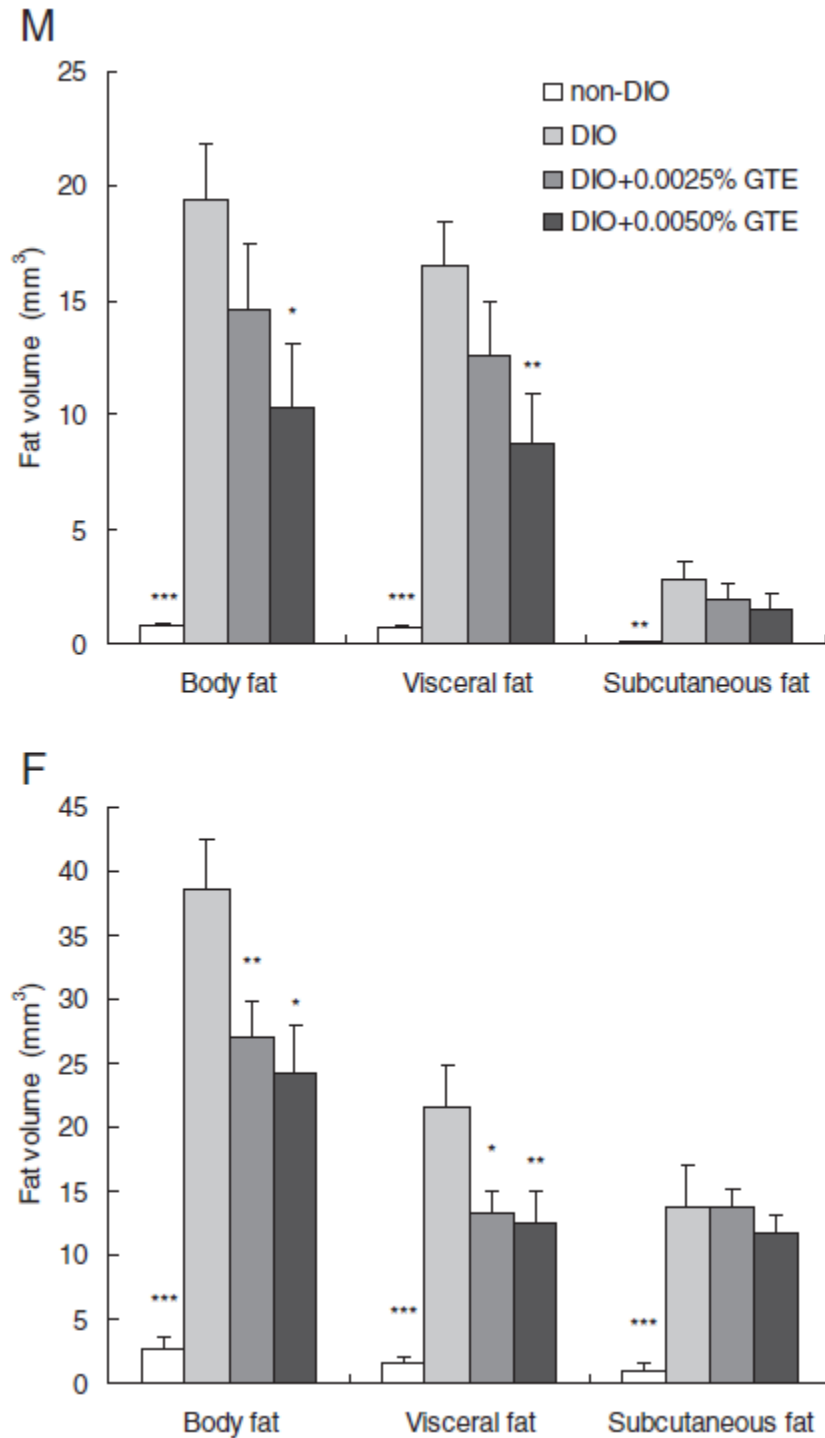
Fig. 11   Effects of green tea extract (GTE) on body fat volume.

Mean body fat volume in male (M) and female (F) zebrafish after 40 days of feeding. Values are means ± SE. ***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$ vs. the diet-induced obese (DIO) group.
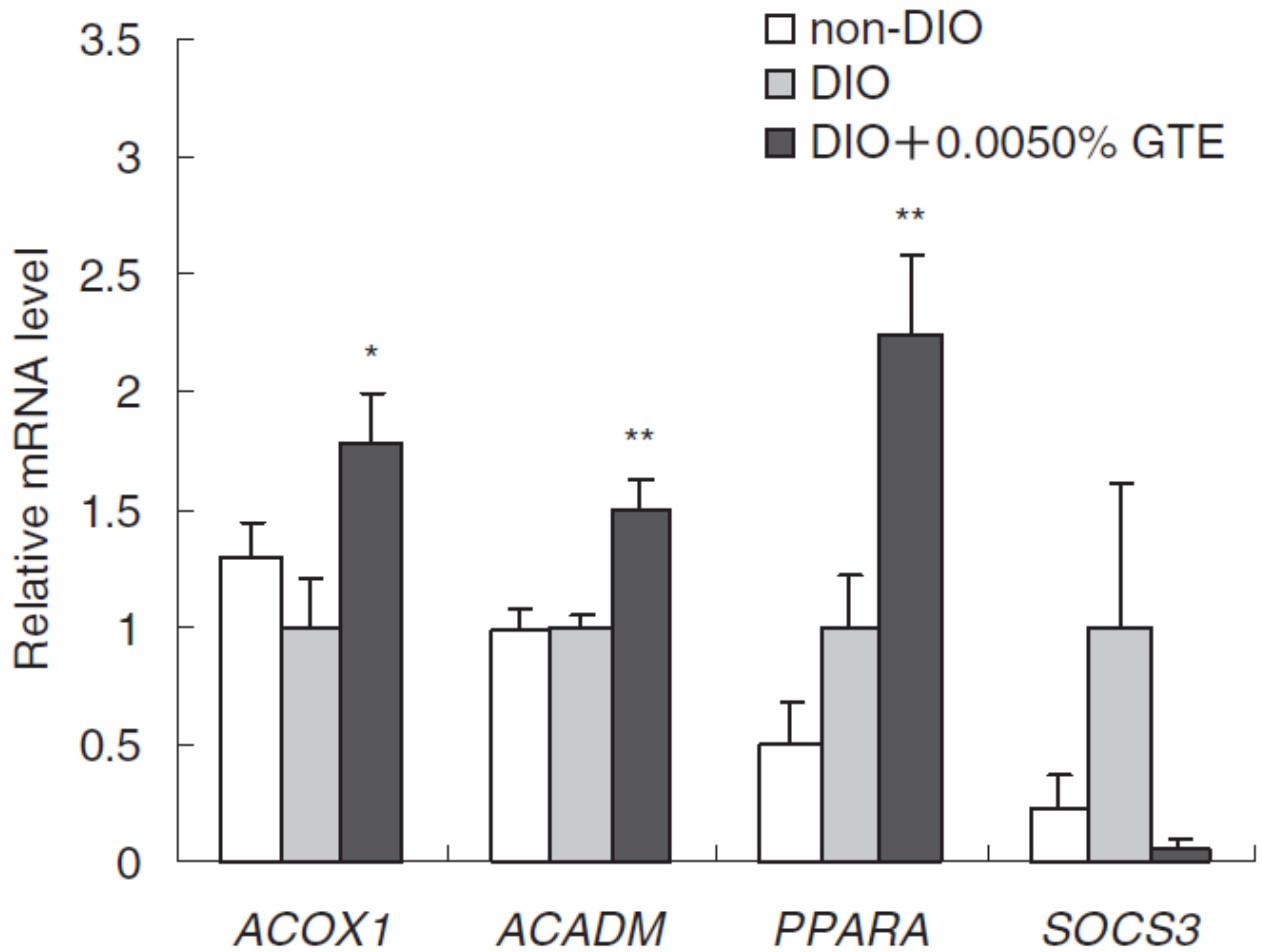
Fig. 12　Effects of green tea extract (GTE) on the expression of lipid metabolism genes in the liver. ACOX1, ACADM, PPARA, and SOCS3 mRNA expression in the liver of female zebrafish on day 21 of feeding. The expression of each mRNA was normalized against PPIA mRNA expression and the corresponding expression in the dietinduced obese (DIO) group. Values are means ± SE. **P < 0.01 and *P < 0.05 vs. the DIO group.
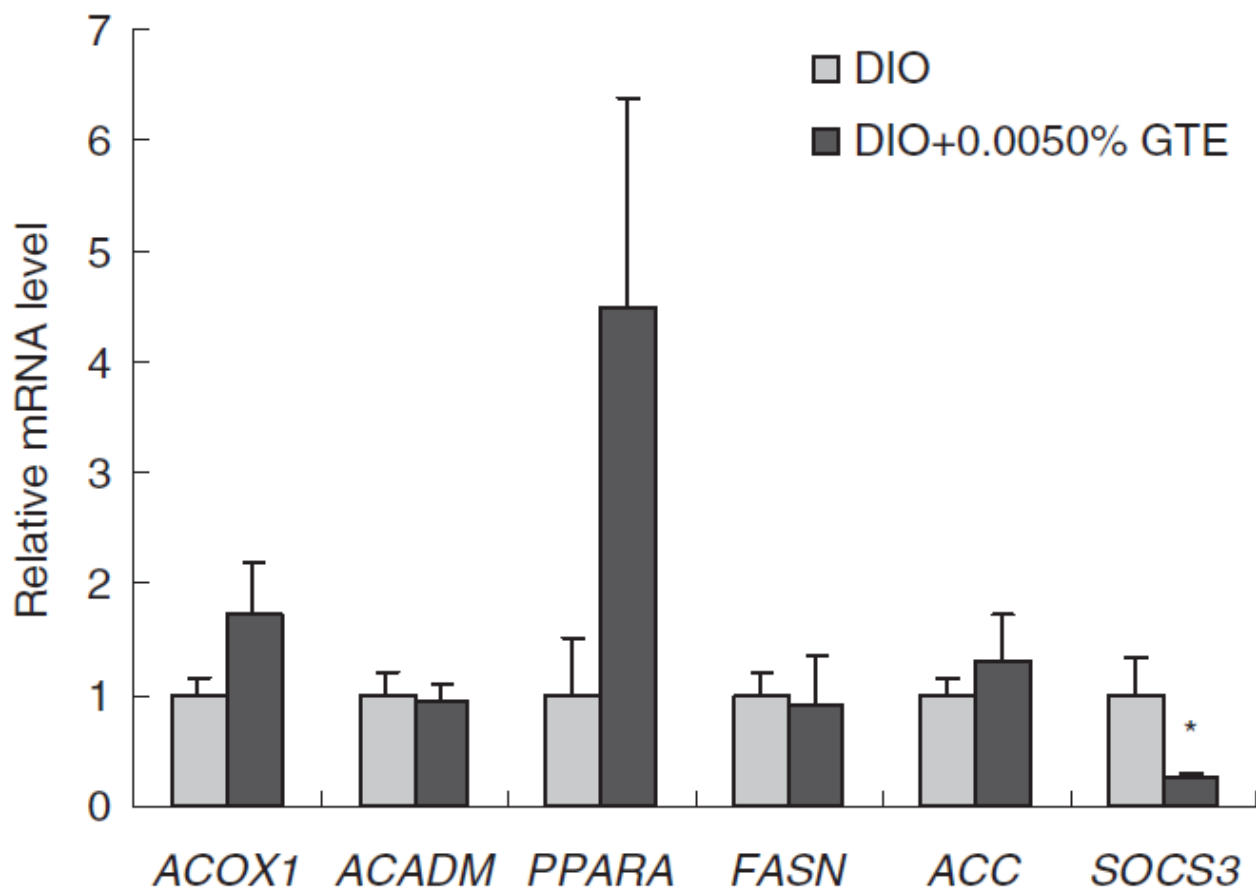
Fig. 13　Effects of green tea extract (GTE) on the expression of lipid metabolism genes in visceral fat.

ACOX1, ACADM, PPARA, FASN, ACC, and SOCS3 mRNA expression in the visceral fat of female zebrafish on day 21 of feeding. The expression of each mRNA was normalized against PPIA mRNA expression and the corresponding expression in the diet-induced obese (DIO) group. Values are means ± SE. *$P < 0.05$ vs. the DIO group.

General Discussion

The results of these studies suggested that zebrafish has a close relationship with the physiology of rodents and humans in relation to the effect of exercises on muscle, nutritional metabolism related to obesity, and body fat accumulation.

In addition, I found in zebrafish a very interesting feature of muscle hypertrophy precisely dependent on exercise quantity occurring in a very short period of time, and induction of obesity with accumulation of body fat in a short period of time due to overfeeding. In rodents, it is difficult to load running exercise without fear stimulation, but it was found that it can be easily studied by using zebrafish. I revealed that zebrafish muscle hypertrophy can be detected with high accuracy at the muscle site (identified by swimming style observation). On the other hands, it was found that zebrafish has commonalities with human in accumulation of visceral fat due to overfeeding and distribution of body fat ,including gender difference. Furthermore, I suggested a new mechanism of this anti-obesity material, tea catechins.

Zebrafish has traditional abundant developmental and genetic biological information and also has features such as high prolificacy and easy genetic manipulation. And this time, the physiological state of exercise and meal of zebrafish where information was limited was clarified. By combining the physiological characteristics of the zebrafish clarified in this study and much biological information originally possessed by zebrafish, it is considered that the zebrafish research model can be utilized for development preventive improvement technology of sarcopenia, locomotive syndrome, and metabolic syndrome. I also believe that this will also accommodate social demands for alternatives to animal testing.

For example, in Japan, disuse syndrome is listed as the leading cause of Needed Support and Long-Term Care. Troubles of locomotorium, especially decreasing of muscle mass and muscle

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strength are serious problems. In humans, age-related muscle mass reduction is known (Nair 2005) and is referred to as sarcopenia, which has been a global challenge (Evans 1993, 1995). Functional deterioration of locomotorium such as muscles is regarded as a health problem as a locomotive syndrome (Nakamura 2008), and research and development to care and improve this is important. In addition, obesity (overweight) is spreading widely, especially in developed countries (WHO 2017). And functional deterioration of muscle and other exercisers as a locomotive syndrome is regarded as a health issue (Nakamura 2008), and development and research of improved techniques for this is important. Obesity caused by accumulation of visceral fat is regarded as metabolic syndrome, leading to various fatal diseases (Itoh 2006) proposed a new concept to capture the flow of events and chain reactions associated with cardiovascular risk: the metabolic domino. In other words, preventing visceral fat accumulation is also very important for health, and research and development of such anti-obesity technology has a great need.

Turning to the point of view and paying attention to the fact that zebrafish is a fish, the research findings on the physiology of zebrafish by this study are the information of zebrafish's Agricultural and Biological Sciences which was insufficient compared to other fish species. In the report of the FAO (Food and Agriculture Organization of the United Nations) (2016), consumption of fishes and shellfishes is growing globally, whereas catch of natural resources by fishery is sluggish over the last 20 or 30 years, while 30% oceanic fisheries resources are said to be "overused or depleted", which is a major problem. Meanwhile, in order to solve this problem, sophistication of aquaculture technology is required, and carp and fun species are the highest in both aquaculture production and volume growth rate globally. Zebrafish itself is not edible, but it is a carp family fish. The findings concerning exercise, nutritional metabolism, muscle and fat of zebrafish, which is one of the most detailed research findings, are thought to be able to lead to study of aquaculture style and

edible part of fish. The multi-faceted research of zebrafish is expected to give further insight into such research and development areas.

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