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ORIGINAL PAPER

Exercise quantity-dependent muscle hypertrophy in adult zebrafish (*Danio rerio*)

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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, we 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 fiver size distributions of exercised fish were larger than those from non-exercised fish. We revealed that exercise quantity, duration/day, and overall duration were correlated with skeletal muscle hypertrophy. The forced exercise model enabled us 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.

Keywords Forced exercise · Muscle mass · Muscle hypertrophy · Zebrafish

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

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. We assume that the reason for this is a lack of appropriate, high-precision animal models.

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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 protein 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, 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, we 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. We 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. We 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 (*D. 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 Brett-style 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 body

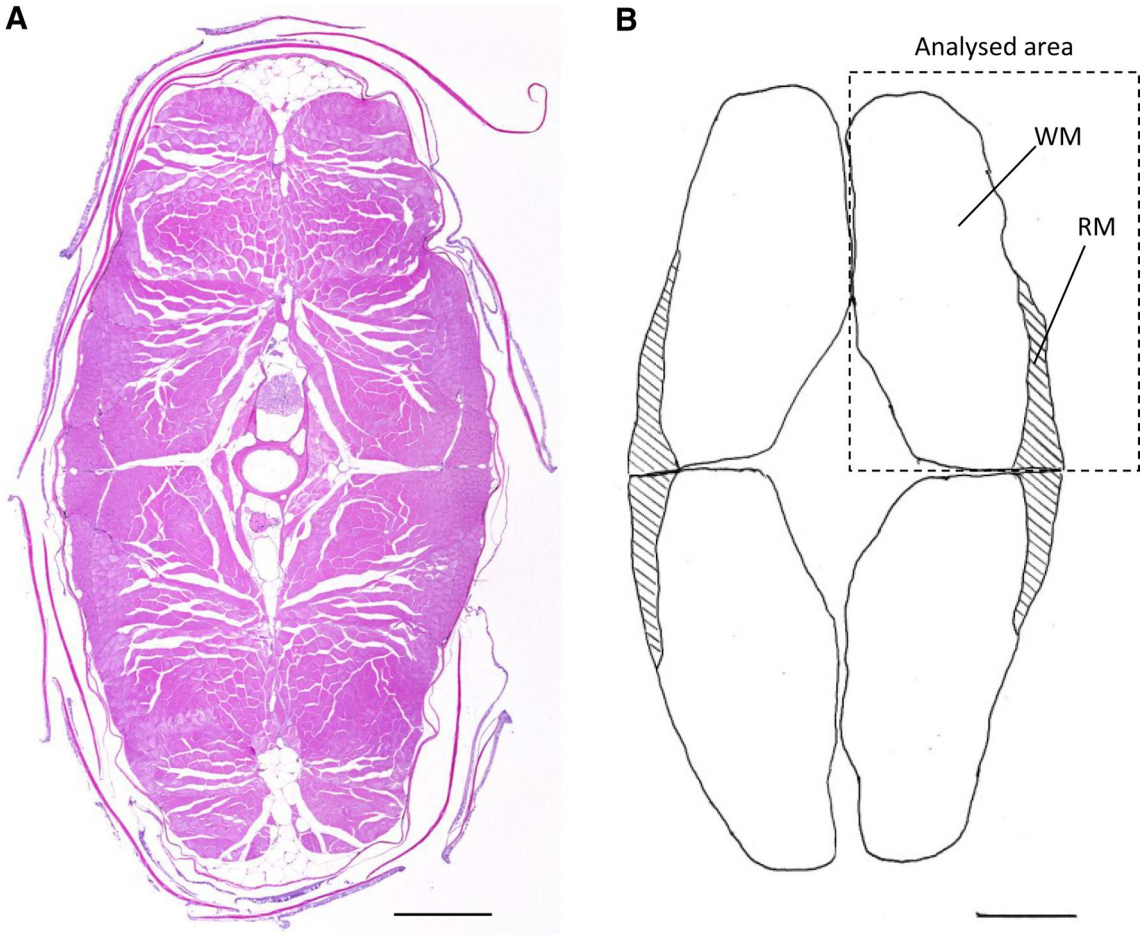

Fig. 1 Zebrafish cross-sectional muscle diagram. **a** Hematoxylin–eosin (HE) stained cross-section of the caudal muscle (Fig. 2, line B). **b** Diagram of the cross-section. RM, red muscle fibers; *WM* white muscle fibers. All muscle area and muscle fiber size measurements were performed in the "analyzed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

weights. 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 (non-exercised 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 swimming 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, we 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.

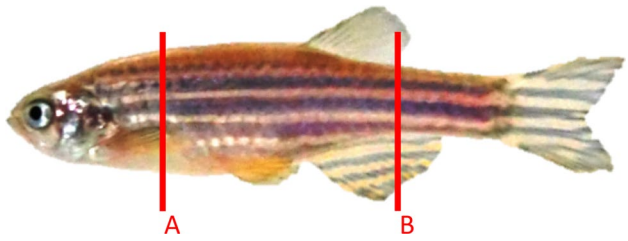

Fig. 2 Cross-sectional position for X-ray micro computed tomography (CT) and histological analysis. Cross-section at lines *A* mid-cranial half muscle and *B* mid-caudal half muscle in zebrafish were measured and analyzed as cranial skeletal muscle and caudal skeletal muscle, respectively, by micro-CT.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

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 cross-sectional 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 non-exercised 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

We 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 mid-caudal 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

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 * $p < 0.05$

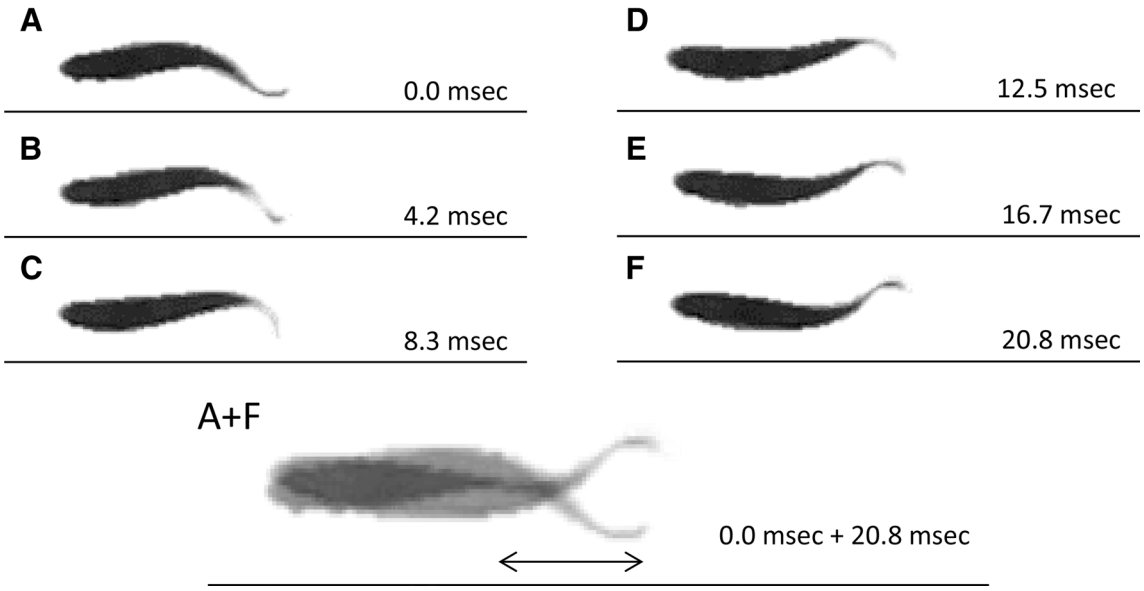

Fig. 3 Consecutive high-speed photographs of zebrafish during fast swimming. All photographs were taken with a high-speed digital camera (240 frames/s) from the dorsal side. **a–f** Consecutive photographs during half of a tail beat cycle (20.8 ms). **g** A + F overlay. The body part indicated by the *two-headed arrow* contracts and bends the body and generates most of the propulsive force

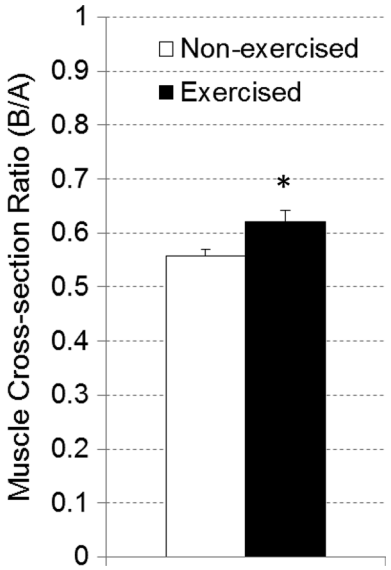

Fig. 4 Muscle hypertrophy in zebrafish after swimming exercise. The ratio of caudal skeletal muscle cross-sectional area (Fig. 2, line B) to cranial skeletal muscle cross-sectional area (Fig. 2, line A) was analyzed by X-ray micro- computed tomography. Values are means ± standard errors. Significant values between non-exercised and exercised groups: $*p < 0.05$

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. 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 non-exercised 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

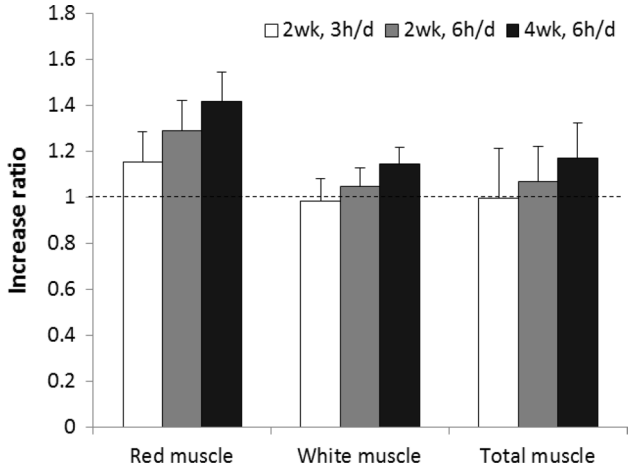

Fig. 5 Effects of changing exercise quantity on increases in the skeletal muscle ratio. The skeletal muscle mass ratio of each muscle type increased with each increase in exercise quantity; 2 weeks, 3 h/day in experiment 3; 2 weeks, 6 h/day in experiment 2; and 4 weeks 6 h/day in experiment 1. The ratio 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 ($p < 0.01$)

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 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 mid-caudal 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

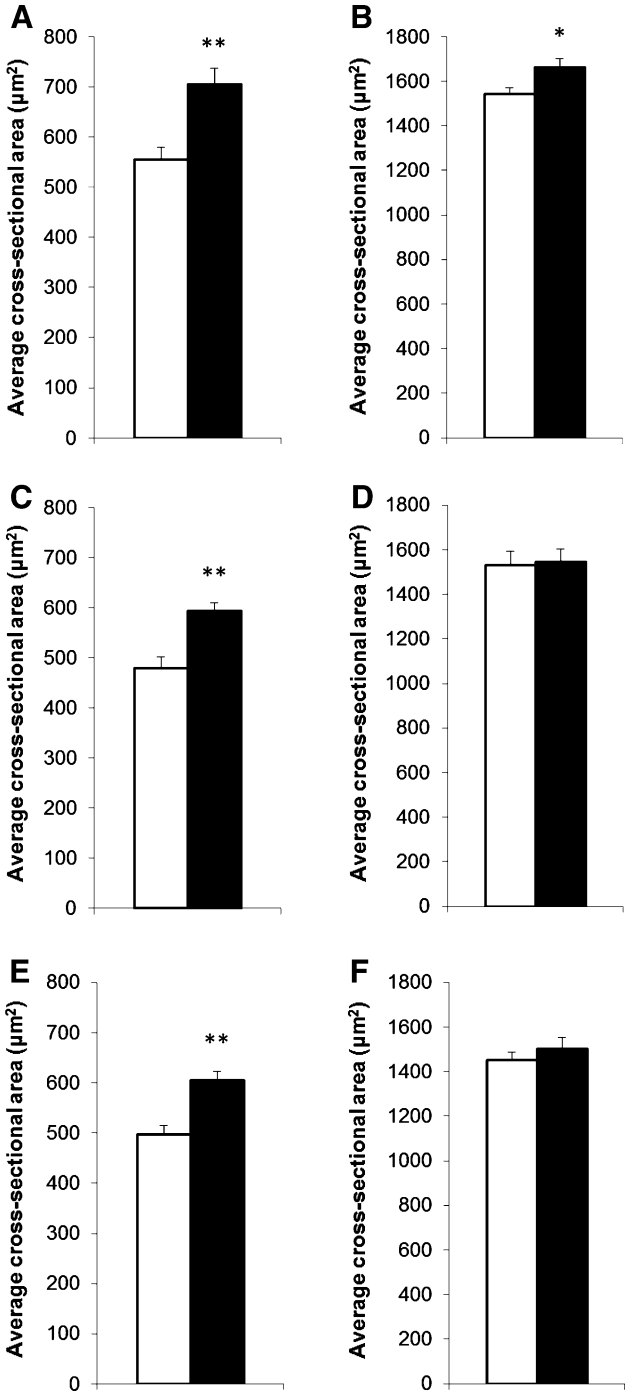

Fig. 6 Morphometric analysis of mean muscle fiber area in zebrafish after swimming exercise. **a** Mean, red muscle fiber cross-sectional area in the non-exercised and exercised groups in experiment 1. **b** Mean, white muscle fiber cross-sectional area in experiment 1. **c** Mean, red muscle fiber cross-sectional 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 ± standard errors. Significant values between non-exercised and exercised groups: $^{**}p < 0.01$, and $^{*}p < 0.05$

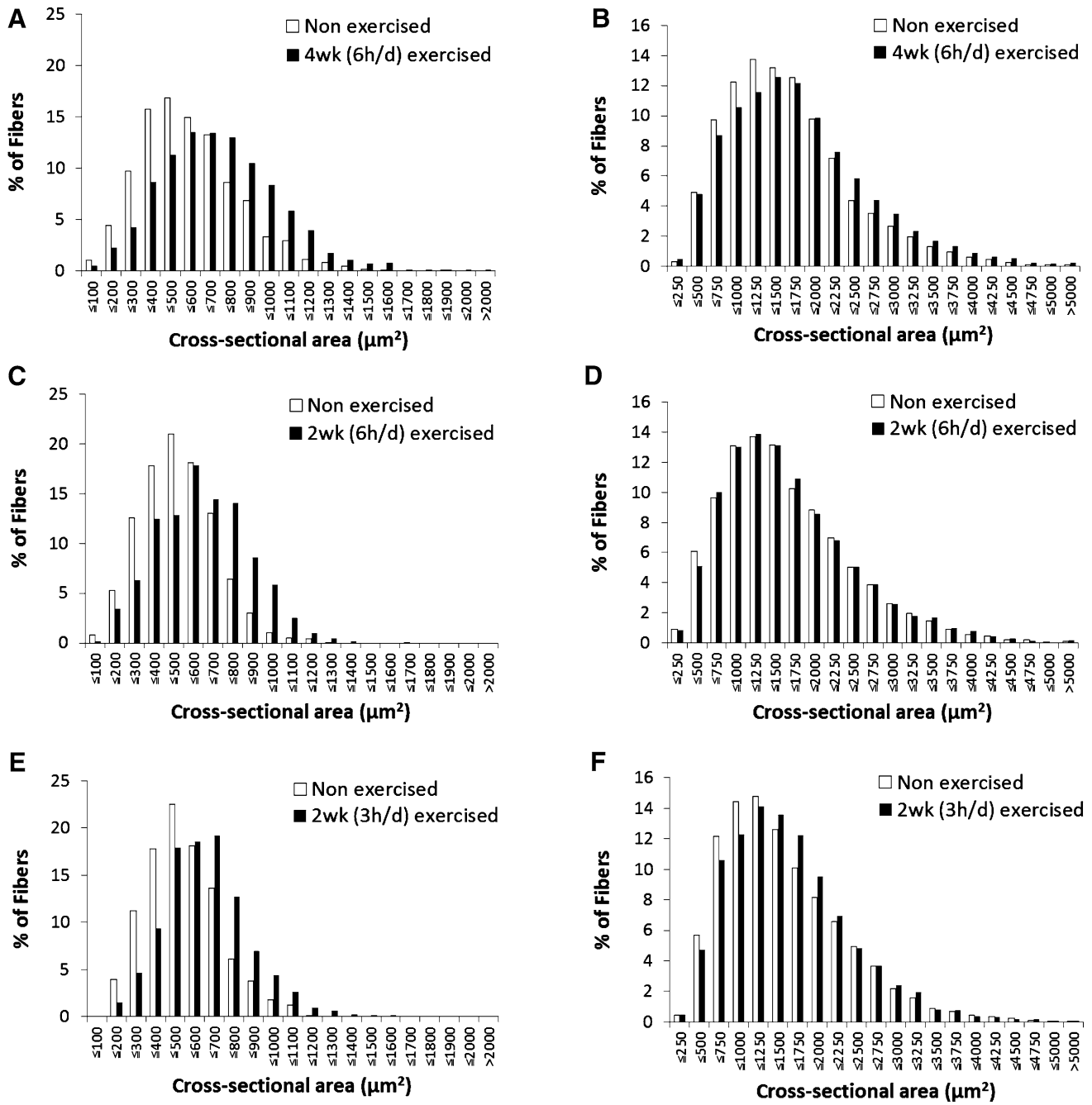

Fig. 7 Histogram of the morphometric analysis results of zebrafish muscle fibers after swimming exercise. **a** Histogram of red muscle fiber cross-sectional area in the non-exercised and exercised groups for experiment 1. **b** Histogram of white muscle fiber cross-sectional area in experiment 1. **c** 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 cross-sectional 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 experiment 3

(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

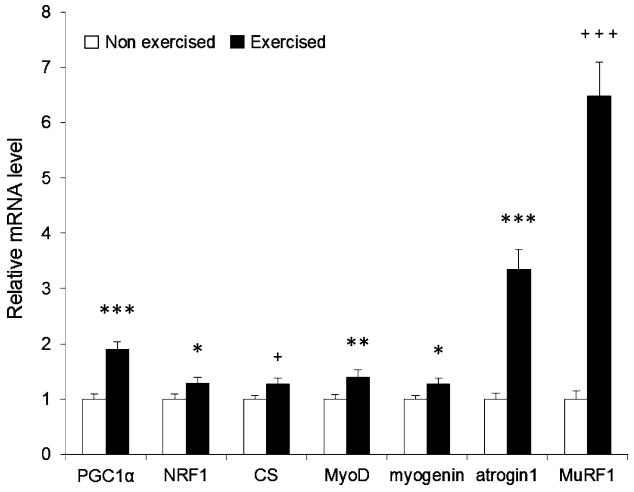

Fig. 8 Effects of a single exercise on skeletal muscle gene expression. Peroxisome proliferator-activated receptor gamma and coactivator 1 alpha (PGC1α), nuclear respiratory factor 1 (NRF1), citrate synthase (CS), myogenic differentiation 1 (MyoD), myogenin, atrogin1, and MuRF1 mRNA expression levels 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

study was sufficient to affect skeletal muscle. Therefore, we 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, we 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, we 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. We 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 1979). 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, we observed high-speed consecutive photographs of zebrafish swimming (Fig. 3) and the appearance (Fig. 2) of zebrafish with reference to Lindsey (1979), 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, we 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, we 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 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.

Our 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.

Our 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 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. We are 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 our 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 our study increased endurance-related white muscle (data not shown), referred to as “pink muscle” in fish (Syme 2005). Thus, the exercise conditions in our 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 our study increased PGC1α, NRF1, and CS gene expression levels. Our 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 our 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 we observed suggest that exercise training affects muscle remodeling and the properties of mitochondria.

Our 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 us to investigate the relationship between exercise quantity and skeletal muscle mass. We 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.

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Compliance with ethical standards

Conflict of interest No author has any competing interest to declare.

Ethical approval All experiments in this study conformed to the regulations approved by the Animal Care and Experimentation Facility Committee of Kao Corporation and with those outlined in The Zebrafish Book (Westerfield 2007) and the Guide for the Care and use of Laboratory Animals 8th edition (National Research Council Committee for the Update of the Guide for the and Use of Laboratory 2011).

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