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Zebrafish and giant danio as models for muscle growth: determinate vs. indeterminate growth as determined by morphometric analysis

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Biga, P. R., and F. W. Goetz. Zebrafish and giant danio as models for muscle growth: determinate vs. indeterminate growth as determined by morphometric analysis. *Am J Physiol Regul Integr Comp Physiol* 291: R1327–R1337, 2006. First published June 1, 2006; doi:10.1152/ajpregu.00905.2005.—The zebrafish has become an important genetic model, but their small size makes them impractical for traditional physiological studies. In contrast, the closely related giant danio is larger and can be utilized for physiological studies that can also make use of the extensive zebrafish genomic resources. In addition, the giant danio and zebrafish appear to exhibit different growth types, indicating the potential for developing a comparative muscle growth model system. Therefore, the present study was conducted to compare and characterize the muscle growth pattern of zebrafish and giant danio. Morphometric analyses demonstrated that giant danio exhibit an increased growth rate compared with zebrafish, starting as early as 2 wk posthatch. Total myotome area, mean fiber area, and total fiber number all exhibited positive correlations with larvae length in giant danio but not in zebrafish. Morphometric analysis of giant danio and zebrafish larvae demonstrated faster, more efficient growth in giant danio larvae. Similar to larger teleosts, adult giant danio exhibited increased growth rates in response to growth hormone, suggesting that giant danio exhibit indeterminate growth. In contrast, adult zebrafish do not exhibit mosaic hyperplasia, nor do they respond to growth hormone, suggesting they exhibit determinate growth like mammals. These results demonstrate that giant danio and zebrafish can be utilized as a direct comparative model system for muscle growth studies, with zebrafish serving as a model organism for determinate growth and giant danio for indeterminate growth.

hypertrophy; hyperplasia; growth hormone; model organisms

ZEBRAFISH POSSESS MANY TRAITS that are desirable for biomedical research, including the external development of clear embryos, short generation interval of 3 mo, and small size that allows for large-scale experiments in small spaces (20, 30). Furthermore, recent large-scale mutagenesis screenings using zebrafish have led to the isolation of genes essential to embryonic development (3, 10, 19). The most important advantage for using zebrafish as a model for any type of biological study is the sequenced zebrafish genome. However, some of the characteristics that have endeared zebrafish to molecular genetics and developmental biology such as small size make them impractical models for traditional physiological studies. Thus the development of a model, like the giant danio, that takes advantage of the same characteristics as the zebrafish, minus the small size, could provide a model beneficial to all areas of biological research.

Muscle growth in vertebrates is defined as being either determinate or indeterminate. Animals such as mammals exhibit determinate growth in which there is a finite size. In contrast, in many fish species, growth is indeterminate, in which there is no fixed size, and some growth may continue throughout the life of the fish (33). The primary difference between these two growth types is the means of muscle fiber growth. In contrast to mammals, fish species exhibiting indeterminate growth increase muscle mass by the recruitment of new muscle fibers (hyperplasia), as well as increasing the size of already existing fibers (hypertrophy) (17, 46). Posthatch hyperplasia, also referred to as muscle recruitment, sets fish apart from other vertebrates in which muscle fiber number is fixed at birth and subsequent growth occurs by hypertrophy only (25). However, not all fish species exhibit an indeterminate growth type, as the regulation of growth is multifactorial, and variations from species to species have been reported. Some recent observations indicate that zebrafish exhibit determinate muscle growth, with very little muscle fiber hyperplasia after the juvenile phase. However, other studies have suggested continued growth post-sexual maturity (15, 41) and remarkable muscle regeneration capabilities (37, 41) in adult zebrafish. In addition, studies in our laboratory have shown only a modest and short-term 20% increase in growth can be achieved by exogenous growth hormone (GH) application in zebrafish (44). Similarly, Morales and coworkers (34) reported a 20% increase in growth rates of GH-transgenic zebrafish expressing an "all fish" GH transgene with β-actin promoter. In contrast, transgenic coho salmon expressing an all-salmon GH construct are extremely fast growing compared with nontransgenic controls, with an average 11-fold increase in weight (8). Thus it appears that there is a finite and low capacity for zebrafish to further increase their size, even in the presence of elevated GH.

In contrast, the giant danio (*Danio aequipinnatus*), a species closely related phylogenetically to zebrafish, appears to exhibit indeterminate growth that is similar to commercially important fish species like trout and salmon. The normal adult size of the giant danio ranges between 10 and 13 cm, which is more than twice the average adult size of zebrafish. Also, compared with the zebrafish, the giant danio exhibited a 129% increase in growth following exogenous GH application (44). Therefore, unlike the zebrafish, the giant danio appears to exhibit indeterminate growth, even though it is very closely related to the zebrafish. The characterization of opposing growth types in closely related fish species such as the zebrafish and giant

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danio could lead to a comparative model system that could be used in studies to further understand mammalian and piscine growth.

Therefore, the present study was conducted to compare and characterize the pattern of muscle growth in zebrafish and giant danios for their future utilization as a direct comparative model system. The major objectives were to investigate the relationship between different types of growth and growth-regulating factors as a means of understanding the difference between determinate and indeterminate growth patterns. To accomplish these objectives, this study utilized traditional morphometric approaches to define growth patterns, as well as evaluated growth responses over time following treatment with GH, a growth-promoting agent.

MATERIALS AND METHODS

Animals. Animal experiments and care procedures were reviewed and approved by Institutional Animal Care & Use committees at both the University of Wisconsin-Milwaukee and Marine Biological Laboratory. All fish were reared under standard conditions, as previously described (58). Adult giant danios were obtained from local breeders and bred in house to ensure correct aging of the sampled juveniles. Zebrafish (Ab) were obtained from the Marine Biological Laboratory zebrafish facility and bred in house. Breeding pairs were chosen randomly to ensure a random sampling population. Larvae were fed sterile, live paramecia (Fish and Frogs, Brighton, MA) four times daily until they reached 6 mm. At 6 mm, larvae were fed both paramecia and live *Artemia nauplii*. Whole larvae were sampled at 1, 2, 3, and 4 wk posthatch. Whole larvae where euthanized by overdose of tricaine methylsulfonate (MS-222). Following euthanasia, larvae were either fixed in 10% buffered neutral formalin for 24 h or flash frozen in Histo Prep (Fisher Scientific) and stored at −80°C. HistoGel (Richard-Allen Scientific, Kalamazoo, MI) was used to maintain position in microcassettes in formalin-fixed samples. Following 24-h fixation, larvae were washed with diethyl pyrocarbonate-treated water and then decalcified in PBS-EDTA for 7 days. Following decalcification, larvae were washed with diethyl pyrocarbonate-treated water and then dehydrated to 70% ethanol. Before embedding, larvae lengths were measured. Larvae were then embedded in paraffin for lateral and cross-sectional orientation.

For the growth trial, 1-yr-old giant danios were obtained from a Tropical Fish Commercial Breeder (Fish2U.com), and 2-yr-old giant danios were obtained from the same local breeder as for the larval study. Zebrafish were obtained as described above and from the Notre Dame University (Ab strain) zebrafish colony. All adult fish were housed in a recirculating Aquatic Habitats Stand Alone system (Aquatic Eco-Systems) at 26°C, maintained under a 14:10-h light-dark cycle, and fed twice daily to satiation with semimoist BioDiet (Bio-Oregon, 46% CP, primary protein source is fish meal supplemented with marine fish oil) and supplemented with live *Artemia nauplii*. Because dietary requirements are not clear for either zebrafish or giant danio, all fish were fed ad libitum the same semimoist diet and supplemented with live Artemia to ensure that fish were receiving a complete ration.

Morphometric analysis. For the analysis of muscle fiber growth pattern in giant danio and zebrafish, morphometric analysis was conducted on formalin-fixed PBS-EDTA decalcified whole larvae cross sections. Two slides per larvae, with a total of four serial sections on each slide, were utilized for all morphometric analyses. A total of six larvae per species at each larval age were utilized for all morphometric analyses. Paraffin blocks were sectioned (10 μm), stained in hematoxylin and eosin, and then mounted in Permount (Fisher) histological media. For each fish, the following parameters were measured and calculated using an Olympus B-MAX fluorescent microscope with Optimas 6 (Media Cybernetics, Silver Spring, MD) image analysis software and the public domain NIH Image J program (http://rsb.info.nih.gov/nih-image/): *1*) A_c, total cross-sectional area, whole epaxial quadrant (shaded gray in Fig. 1); *2*) N_f, total number of fibers within the epaxial quadrant; *3*) A_t, sum of fiber areas calculated based on the assumption that the fibers are sectioned in circular manner with a diameter equivalent to their width; and *4*) A_f, mean fiber area (A_t/N_f).

A_c of muscle fibers was determined by measuring the total area of one entire epaxial quadrant of the myotome (Fig. 1) (14, 27). The total numbers of fibers within each epaxial quadrant was counted using two techniques, depending on size and stage of the myotome. For small myotomes, with undistinguishable segments of the epaxial myotome, the number of fibers was calculated for the entire epaxial quadrant. In larger myotomes with distinct segmentation, three segments (*a–c*; Fig. 1) were identified, and fiber numbers were collected within each sector. The sums of all segments were calculated for total quadrant fiber number. Cellularity was calculated as the number of fibers divided by the A_c. Fiber diameter was measured on 50 white fibers from five predetermined areas of the myotome. Fiber area was calculated based on the assumption that the fibers are circular with a diameter equivalent to their width, (diameter/2)2*π.

Changes in A_c and A_t over time were compared within and between each growth type. The relative contribution of hyperplasia and hypertrophy was estimated using the method described by Alami-Durante et al. (2). Several studies have demonstrated that recently recruited muscle fibers, responsible for hyperplasia, are relatively small in size (<20 μm^2) (12, 35, 46, 52). Therefore, fibers with diameters >20 μm were classified as representing hypertrophic growth, and those <20 μm were considered indicative of hyperplastic muscle growth. Epaxial quadrants showing hyperplastic growth were classified as exhibiting stratified or mosaic hyperplasia based on the placement of small fibers within the myotome, according to Rowlerson and Veggetti (42) and Patruno et al. (36).

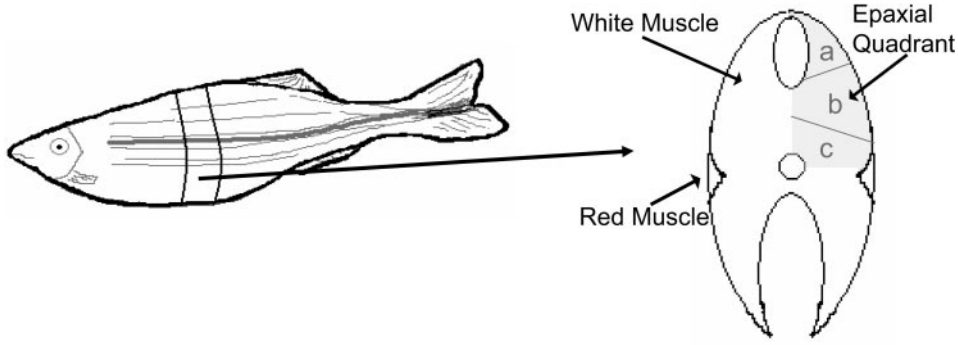


Fig. 1. Diagram of a giant danio showing cross-sectional area (CSA) used for morphometric analyses. The epaxial myotome quadrant is shaded gray. In 4-wk posthatch larvae, the epaxial myotomes were counted in segments, as represented by letters *a*, *b*, and *c*.

Histochemical analysis. Cross sections of flash-frozen samples were sectioned on a crystat (20 μm) and mounted on positively charged slides. Cross sections from larval and adult zebrafish and giant danio were used to demonstrate myofibrillar ATPase activity using previously described staining methods (18), with modifications (45). This method involves the selective inhibition of fiber types by preincubation at varying levels of alkalinity or acidity. Sections were preincubated in an acid or alkaline buffer without prior fixation, as fixations cause substantial inactivation of myofibrillar ATPase activity in all of the fiber types in fish (18). To compare results obtained from zebrafish and giant danios at different life stages, sections were treated simultaneously in the same incubation medium. Incubation times in ATP solutions were increased to 30 min for both acid and alkaline preincubations because fish ATPase appears to be more labile.

Growth performance analysis. To assess the differences between early life growth, larval fish length was measured beginning at hatch and every week for 4 wk using a dissecting microscope and a micrometer. A 17-wk growth trial was conducted to evaluate the growth response to hormone treatment in adult giant danio and zebrafish. For this trial, a sustained-release, recombinant bovine GH (rbGH; Posilac, lot no. 03F06/2D, Monsanto) preparation was injected (intraperitoneal, 120 μg/g body wt) every 4 wk for 4 mo using a 1-ml syringe equipped with a 29-gauge needle. The frequency and amount of rbGH used in this study were chosen based on previously reported results (43, 44). The amount of rbGH was calculated based on total GH content of the Posilac preparation, and control fish were injected with the excipient alone at the same volume as calculated if GH were present. Two giant danio age groups, 1 and 2 yr of age, were used to evaluate an age-dependent growth response to GH. Previous studies have demonstrated that adult zebrafish do not respond to rbGH with increased overall growth (44); therefore, only one age group of zebrafish (2 yr) was utilized for this trial. Zebrafish were separated by sex to ensure that growth could be assessed on a male vs. female basis to separate the effect that egg production might have on female growth. Weight and lengths of fish were taken weekly to monitor growth. At the termination of the trial, one entire side of the myotome was flash frozen in Histo Prep, sectioned on a cryostat, and stained. Fiber number and area were calculated from white muscle sections as described for larval fish, with some modification. For each cross section, four 3-mm^2 sections were outlined within the white fiber layer only. Within these sections, the number of fibers was counted, and their total area was calculated. Fibers were only counted if >50% of their area was within the box. The presence and extent of hyperplasia were measured and compared between species and treatment groups.

Data analysis. Morphometric, length, and weight data were analyzed using two-way analysis of variance (SigmaStat, SPSS) with factors being time and species. Pairwise multiple-comparison tests were conducted by the Holm-Sidak method to compare between and within groups. Normality of sample distribution was tested by Normal Quantile Plot (Q-Q Plot), and residuals were plotted against predicted values to evaluate dependency between the means and variances and test the assumption of homogeneity of variances. When normality failed, data were separated by species, and normality was tested within species to ensure that the skewness was based on size difference between the two species and not internal sample skewness. For adult fiber size and number, *t*-tests were conducted within each species. Results are reported as least square means ± SE. Results were considered significant when $P < 0.05$.

RESULTS AND DISCUSSION

Larval growth. Giant danio adults reach a much larger size (10 cm) compared with zebrafish (6 cm). Figure 2 shows the differences between growth in length for the first 4 wk of posthatch larval development in giant danio and zebrafish.

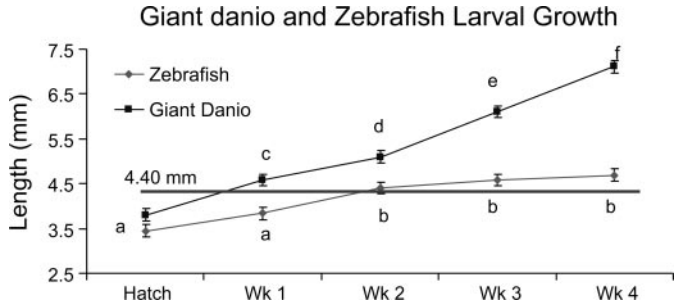


Fig. 2. Growth results of giant danio and zebrafish larvae from hatching to 4 wk posthatch measured as length (*L*) (mm). The vertical line represents the mean *L* of 1 wk posthatch giant danio larvae, 4.71 mm. $N = 6$ fish/time. [a-f]All data points with different letters represent significance at $P < 0.0001$.

There was no difference in size between giant danio and zebrafish larvae at hatch ($P = 0.08$; 3.803 ± 0.133 and 3.448 ± 0.133 mm, respectively). Zebrafish larvae size increased 38.9% compared with 84.2% in giant danios from hatch to 4-wk posthatch. Interestingly, it took zebrafish larvae 3.5 wk to reach the size of 1-wk posthatch giant danio. Giant danio larvae were larger ($P < 0.0001$) than zebrafish at all time periods measured, except hatching. Within species, zebrafish larvae increased ($P < 0.0001$) in length from *week 1* to *week 2*, but there was no difference ($P > 0.10$) between 2, 3, or 4 wk (4.400 ± 0.133, 4.582 ± 0.133, and 4.692 ± 0.133 mm, respectively). In comparison, giant danio increased ($P < 0.0001$) in length every week, beginning at *week 1* (1 wk, 4.583 ± 0.133; 2 wk, 5.100 ± 0.133; 3 wk, 6.105 ± 0.133; 4 wk, 7.017 ± 0.133 mm).

The present study is the first comparative report of early larval growth in these species. Eaton and Farley (11) previously described zebrafish larval growth in relation to food availability. In that study, zebrafish grew at a rate of ~0.05 mm/day for the first 45 days of life with maximum food available. Here we report a similar average daily growth rate, 0.04 mm/day, for the first 28 days of life. In comparison, the giant danio exhibited an average daily growth rate of 0.1 mm/day. Eaton and Farley reported that, at 6 mm, zebrafish are capable of feeding on live *Artemia nauplii*. In our study, all larvae were fed sterile paramecia four times per day to ensure an unlimited food supply. At 3 wk, when giant danio larvae reached 6 mm, they were fed both paramecia and *Artemia nauplii*. Zebrafish larvae were not given *Artemia* because they never reached 6 mm in length in this study. It is possible that the higher growth rate observed in giant danio larvae could be a result of the availability of higher quality food. However, giant danio larvae consistently grew larger every week of the trial, even before the introduction of *Artemia*.

Goolish and coworkers (16) also reported similar mean lengths at hatch (3.53 ± 0.03 mm at 4 days postfertilization) in zebrafish larvae. However, they reported a larger increase in growth at 21 days postfertilization (7.30 mm) than reported here (4.69 mm). It is possible that the difference in growth could be due to different rearing temperatures, as larvae in this study were reared at 25°C compared with 28°C as reported by Goolish et al. Barrionuevo and Burggren (5) reported differential zebrafish larval growth at different rearing temperatures, with growth being significantly less at 25°C compared with 28 and 31°C (4.1 vs. 7.2 and 8.7 mm, respectively). Another

recent study also demonstrated larger larval size at 21 days postfertilization [6.4 mm (Ref. 4)] in zebrafish, but the rearing temperature was not reported. However, that study utilized wild-type zebrafish, while the study presented here used the AB strain of zebrafish. It is also possible that the discrepancies in larval growth between the present study and others is the utilization of different strains of zebrafish. It is well established that teleosts can exhibit species- and strain-specific growth attributes (26, 29). Therefore, it is possible that the differences in reported larval growth are a result of differences in rearing temperature, zebrafish strain, or a combination of these parameters.

Muscle morphometrics. Morphometric analysis revealed that the overall area of the epaxial (one quadrant) myotome increased 570% in the first 4 wk posthatch in the giant danio (2,796.72 μm^2 at 1 wk vs. 18,757.13 μm^2 at 4 wk) and 575% in the zebrafish (1,689.49 μm^2 at 1 wk vs. 1,1414.81 μm^2 at 4 wk). For the first 2 wk of posthatch growth, there were no differences detected in the overall area of the epaxial myotome between the giant danio and zebrafish ($P = 0.40$). At 3 wk posthatch, the giant danio myotome grew 51% from 4,491.50 to 6,814.55 μm^2 at *weeks 2* and *3*, respectively. From *week 3* to *4*, the giant danio myotome increased ($P < 0.001$) 175% from 6,814.55 to 18,757.13 μm^2, respectively. Comparatively, the zebrafish myotome did not increase from *week 2* to *3* ($P = 0.92$) but did increase 290% from 2,923.31 to 1,1414.81 μm^2 from *week 3* to *4*, respectively ($P < 0.0001$). The giant danio epaxial myotome was consistently larger than the zebrafish myotome at *weeks 3* and *4* (Fig. 3*A*). When the quadrant area was corrected for larvae length, there was no difference between epaxial myotome growth in giant danio and zebrafish (Fig. 3*B*) from 1 wk to 4 wk of larval growth. The only detectable difference was an increase from *weeks 3* to *4* in both species of fish, suggesting that the myotomes of both zebrafish and giant danio are growing proportionally with length. However, linear regression analysis, performed following logarithmic transformation of the data, revealed no significant correlation between myotome cross-sectional area and length of zebrafish larvae [$P = 0.737$, $r^2 = 0.00898$, ln(area) = 0.688∗ln(length) + 7.182; Fig. 3*C*]. In contrast, a linear relationship was demonstrated between myotome area and length in giant danio [$P < 0.001$, $r^2 = 0.838$, ln(area) = 3.899∗ln(length) + 2.014; Fig. 3*C*], suggesting overall growth in length and width early in giant danio larval growth.

Zebrafish larvae only increased in total length from *week 1* to *2*, and their epaxial myotome increased from *week 3* to *4*, suggesting that they are growing in width only from *week 3* to *4* and are not actively growing in length or width between *weeks 2* and *3*. In comparison, the giant danio appear to be growing in both length and width, as they increased in length throughout the trial and increased epaxial myotome starting at *week 2*. This time period coincides with the acceptance of live food in both larval species. The first onset of live feeding begins approximately between 4 and 9 days posthatch. At 2 wk posthatch, all larvae are eating live paramecia, and rapid growth has begun. The linear relationship between myotome area and length in early larval growth observed here is consistent with results reported for carp (*Cyprinus carpio*) larvae (2). These results suggest that overall growth in the giant danio is more efficient, as they exhibit linear growth starting at 2 wk posthatch, compared with the zebrafish, in which growth appears to plateau after 2 wk posthatch.

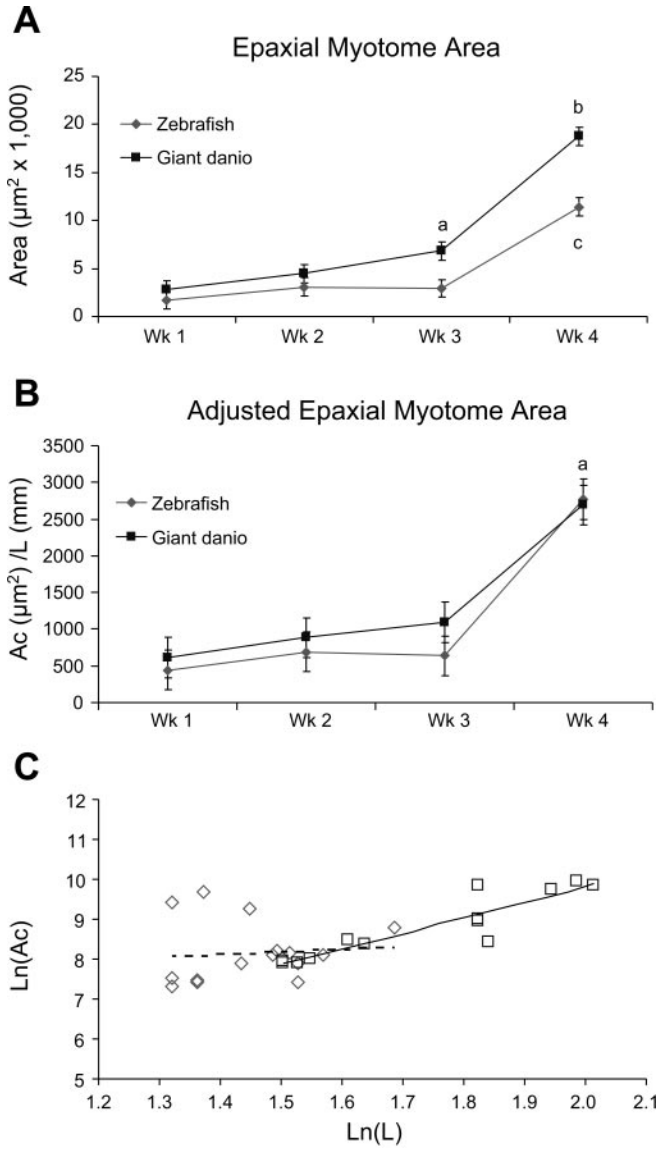


Fig. 3. *A*: total epaxial myotome area in zebrafish and giant danio, 1–4 wk posthatch larval growth. Area, in μm, was measured for one-half (or one side) of the entire epaxial myotome using ImageJ Software. *B*: epaxial myotome area corrected for by *L*, in mm. [a-c]Different letters represent differences at $P < 0.0001$. *C*: logarithmic plot showing relationship between epaxial myotome area and total larvae *L* in giant danio (□) and zebrafish (◇). Corresponding equations are as follows: giant danio, ln area = 2.014 + 3.899∗ln *L*, $r^2 = 0.838$, $P < 0.001$; zebrafish, ln area = 7.182 + 0.688∗ln *L*, $r^2 = 0.00898$, $P = 0.737$. A_c, total CSA.

In larger larvae myotomes that were divided into segments, no difference was detected for the size of segment ($P = 0.110$) or number of fibers within each segment ($P = 0.076$) for either species at any sampling period (data not shown). In other fish species, differences between these segments are typically seen in young larvae, with the greatest differences seen in the apical region (*segment a*) (40, 49). Similar to myotome area, no difference ($P > 0.05$) in total number of muscle fibers present in the epaxial quadrant was detected between zebrafish and

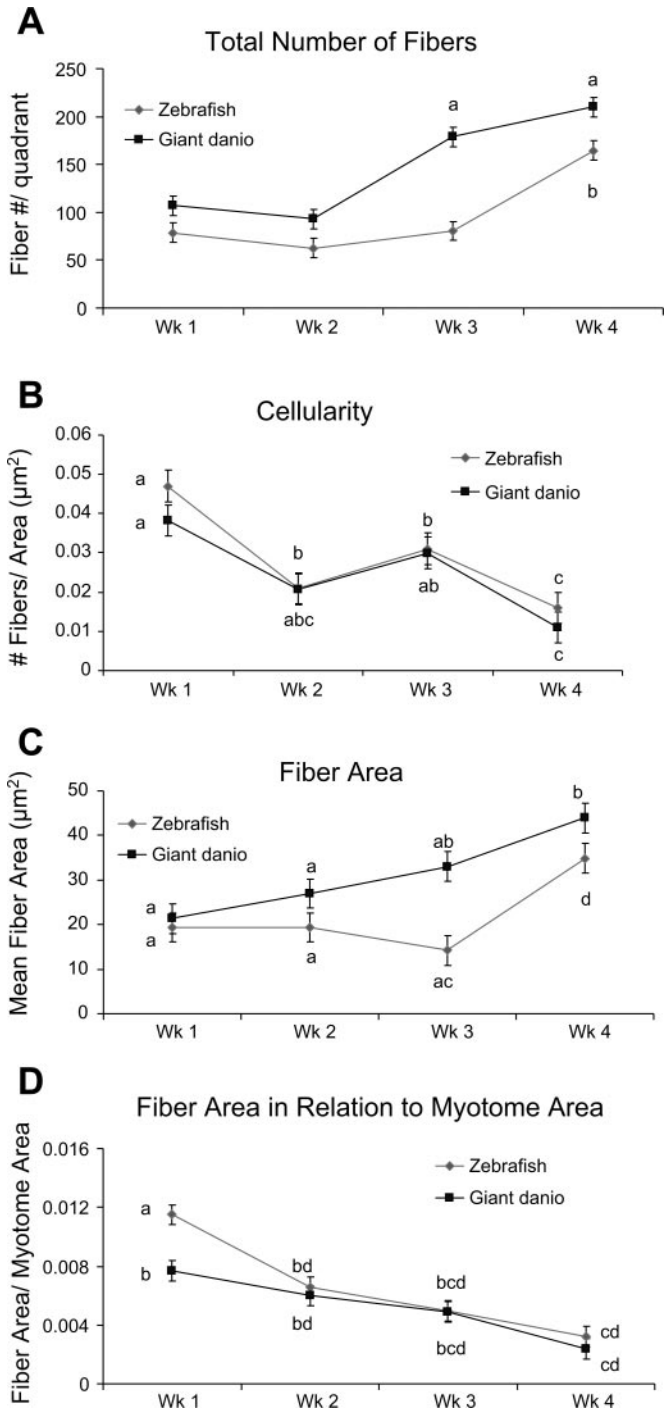


Fig. 4. *A*: total number of fibers (N_f) per epaxial quadrant in zebrafish and giant danio, 1–4 wk posthatch larval growth. The numbers of fibers were counted for one-half of the entire epaxial myotome. *B*: cellularity was calculated as number of fibers/CSA for zebrafish and giant danio at 1–4 wk posthatch. *C*: fiber diameters were calculated for 50 randomly chosen white (fast) fibers within the epaxial myotome. Diameters (*d*) were converted to area, (*d*/2)^2*π, with the assumption that all fibers were circular is shape and the diameters were measured at the longest distance of each fiber. *D*: fiber areas corrected for total epaxial myotome area. [a-d]Letters represent significance at $P < 0.0001$.

giant danio at 1 or 2 wk of age (Fig. 4*A*). Giant danio larvae did, however, exhibit more ($P < 0.0001$) muscle fibers per epaxial quadrant than zebrafish larvae starting at 3 wk of age. Similar to sea bream larvae (40), the total number of epaxial fibers increased rapidly by approximately doubling between 14 and 21 days of development in the giant danio. This increase in fiber numbers is not as rapid as seen in sole, in which the total number of white fibers has been reported to increase from 36 to 810 in the first 21 days of development (50). In comparison, zebrafish larvae did not increase myotome fiber numbers until *week 4*, while giant danio larvae increased the total fiber number at *week 3*. These results correspond with the data on epaxial myotome area, suggesting a relationship between myotome cross-sectional area and fiber number. When cellularity (fiber number/cross-sectional area) was calculated, no difference was detected between zebrafish and giant danio, suggesting similar relationships between myotome area and fiber number in zebrafish and giant danio (Fig. 4*B*).

Fiber area was calculated based on the fiber diameters of 50 white fibers within the epaxial myotome of both species at all times. No difference was detected ($P > 0.10$) in mean fiber area between zebrafish and giant danio larvae at 1 or 2 wk of larval growth. Consistent with fiber number and epaxial myotome area, giant danio mean fiber areas were larger ($P < 0.05$) at *weeks 3* and *4* compared with zebrafish (Fig. 4*C*). When mean fiber area was corrected for overall myotome area, zebrafish exhibited a larger ($P < 0.001$) ratio (fiber area/myotome area) than giant danio at *week 1*, due to the greater difference in myotome area (1,698 ± 950 vs. 2,796 ± 950 μm^2) compared with the difference in mean fiber area (19 ± 3.3 vs. 21 ± 3.3 μm^2) in zebrafish and giant danio, respectively (Fig. 4*D*). These combined results suggest that muscle growth in early zebrafish and giant danio larval development may be due to both hypertrophic and hyperplastic fiber growth, as similar relationships were found between fiber number and fiber area compared with myotome area. In the guppy, early postnatal growth is primarily due to hypertrophic growth, as mean fiber diameter progressively increases and the proportion of small-diameter fibers drops (49).

Total myotome area, mean fiber area, and total epaxial fiber number all exhibited similar relationships with overall larvae length (Fig. 5), with a significant positive relationship in giant danio ($P < 0.01$; Fig. 5*A*) and no significant relationship in zebrafish ($P > 0.10$; Fig. 5*B*). In giant danios, myotome area increased faster (2.01) relative to total larvae length than the total fiber number (1.89) and mean fiber area (0.907). However, total fiber number increased faster than mean fiber area, indicating hyperplastic muscle growth. Hyperplasia accounted for 67% of muscle growth in the giant danio, according to calculations based on total fiber number (1.898) in relation to calculated overall area (2.805). These results are consistent with the relationships reported between muscle fiber growth and somatic growth in carp larvae (2). In contrast, in zebrafish larvae, hyperplasia accounts for only 47% of muscle growth (4.433/9.258). Most fish exhibit both hypertrophic and hyperplastic muscle growth, but the level of hyperplastic growth is determined by factors such as developmental stage, environmental condition, and physiological stage (7, 28, 39, 46, 48, 51, 55–57, 59, 60). It has also been reported that hyperplastic growth is particularly important in fish species, which obtain a larger body size, and when large increases in body size occur rapidly (49). It has previously been reported that body size has an important impact on the maximum number and size of

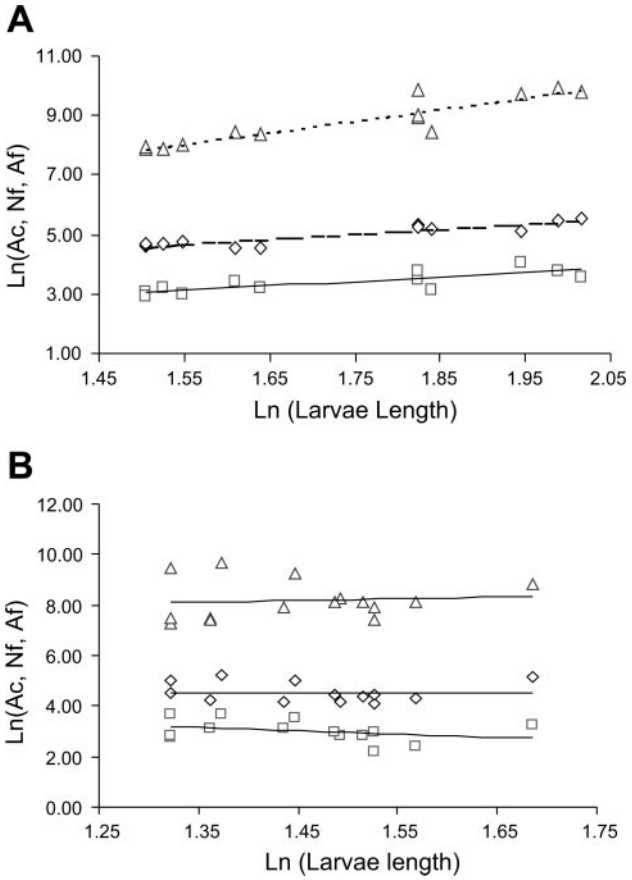


Fig. 5. Logarithmic plots showing for one epaxial quadrant of muscle the relationships between total epaxial myotome area (A_c; △; μm^2), N_f per epaxial quadrant (◇; μm^2), mean fiber area (A_f; □; μm^2), and total larvae L (mm). *A*: giant danio relationships. Corresponding equations are as follows: $A_c = \ln A_c = 2.014 + 3.899*\ln L$, $r^2 = 0.838$, $P < 0.001$; $N_f = \ln N_f = 1.898 + 1.781*\ln L$, $r^2 = 0.831$, $P < 0.001$; $A_f = \ln A_f = 0.907 + 1.438*\ln L$, $r^2 = 0.627$, $P = 0.001$. *B*: zebrafish relationships. Corresponding equations are as follows: $A_c = \ln A_c = 7.182 + 0.688*\ln L$, $r^2 = 0.00898$, $P = 0.737$; $N_f = \ln N_f = 4.433 + 0.0582*\ln L$, $r^2 = 0.000260$, $P = 0.954$, $A_f = \ln A_f = 4.825 - 1.248*\ln L$, $r^2 = 0.100$, $P = 0.250$.

muscle fibers (27, 54, 57). In the context of this study, the giant danio is consistent with a fish species that increases in body size quickly and obtains a larger body size compared with the zebrafish. This is also consistent with more muscle growth being due to hyperplastic growth in the giant danio compared with the zebrafish.

Fiber area distributions are shown in Fig. 6 for both zebrafish and giant danio at *weeks 1–4* of larval growth. The patterns at *weeks 1* and *2* appear very similar between zebrafish and giant danio, which is consistent with all other muscle morphometric analyses reported here. At *week 3*, the giant danio appears to begin to develop larger fibers, and by *week 4*, the prevalence of small-diameter fibers is lower (32%, >20 μm^2). In comparison, at *week 3*, the zebrafish still exhibits many small-diameter fibers (≤ 20 μm^2) compared with larger fibers (>20 μm^2). At *week 4*, the zebrafish begins to develop larger fibers (>40 μm^2) but still maintains small-diameter fibers (36%, <20 μm^2). The guppy also exhibits a similar progressive increase in mean fiber area, with the proportion of fibers <25 μm^2 falling to 2% at 27 days posthatch (49). In comparison, 4-wk-old giant danio and zebrafish in this study still exhibited a relatively high proportion of small-diameter fibers. This progressive increase in mean fiber area that demonstrates postnatal growth is primarily by hypertrophy, which is consistent with other small species where hypertrophy is the main growth mechanism (52). However, both giant danio and zebrafish maintain small-diameter fibers at least until 4 wk of age, indicating a longer period of posthatch myogenic proliferation or stratified hyperplasia.

In the gilthead sea bream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax* L.), mean fiber diameter increased only modestly in the first 46 days posthatching, but changed drastically by 60 days (40, 48). The percentage of very small-diameter fibers (>5 μm^2) is very low at hatching in the sea bream, suggesting that the formation of new fibers progresses slowly posthatch and myogenic growth is accomplished primarily by hypertrophy. In 4-wk-old giant danio, the fiber distribution appears to become bimodal, with a mean fiber diameter of 44 μm^2, while the mean fiber diameter of 4-wk-old zebrafish was 34 μm^2 and approaching a bimodal distribution. The progression of fiber growth in the giant danio is rapid in

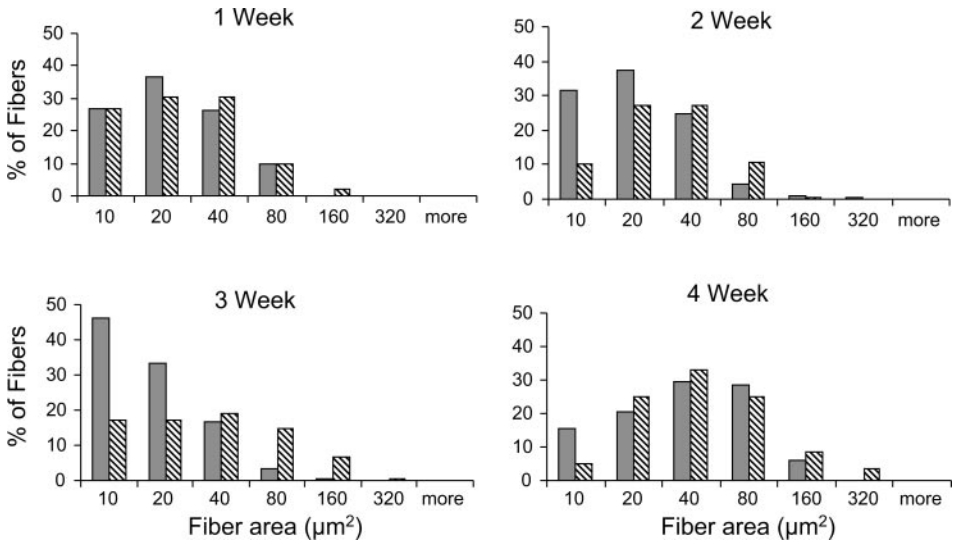


Fig. 6. Larval fiber area distribution. *x*-Axis = fiber size distribution (μm^2); *y*-axis = percentage of fibers. Solid bars represent zebrafish; hatched bars represent giant danio.

early larval growth, and the presence of small-diameter fibers is consistent with the rapid overall growth during this stage of development. Hyperplastic growth in other species was found to be related to fast growth, while hypertrophy is important during slow growth (21).

Both zebrafish and giant danio appear to exhibit some degree of stratified hyperplasia at all larval stages measured. Stratified hyperplasia is essentially a continuation of myogenic hyperplasia and can be defined by small-diameter fibers being predominantly located in the proliferation zone below the skin (42). The first phase of hyperplastic growth in larval development is thought to be a continuation of embryonic myogenesis, since it occurs along the proliferation zone (Fig. 7, *C* and *D*) and is responsible for completing the formation of the distinct muscle fiber-type layers (42). Mosaic hyperplasia is the last phase of hyperplastic growth that occurs in fish that grow to a large final size (38). This process gives rise to the typical mosaic appearance of large fish muscle. Smaller diameter fibers were present in adult giant danio (1.5 yr) myotome, resulting in this characteristic mosaic appearance (Fig. 7*G*). The presence of small-diameter and immature fibers in the apical region of the epaxial myotome is indicative of mosaic hyperplastic growth (36). At 4 wk and 1.5 yr of age, small-diameter fibers predominate the fiber type in the apical region of the epaxial myotome of giant danio (Fig. 7, *E* and *H*), but few to no small-diameter fibers are seen in the zebrafish apical region (Fig. 7, *F* and *I*). This is consistent with previous reports showing the reduction or lack of mosaic hyperplasia in zebrafish and other small fish (31, 47, 49). However, this is the first report of differential hyperplastic patterning between two closely related fish species during early larval growth.

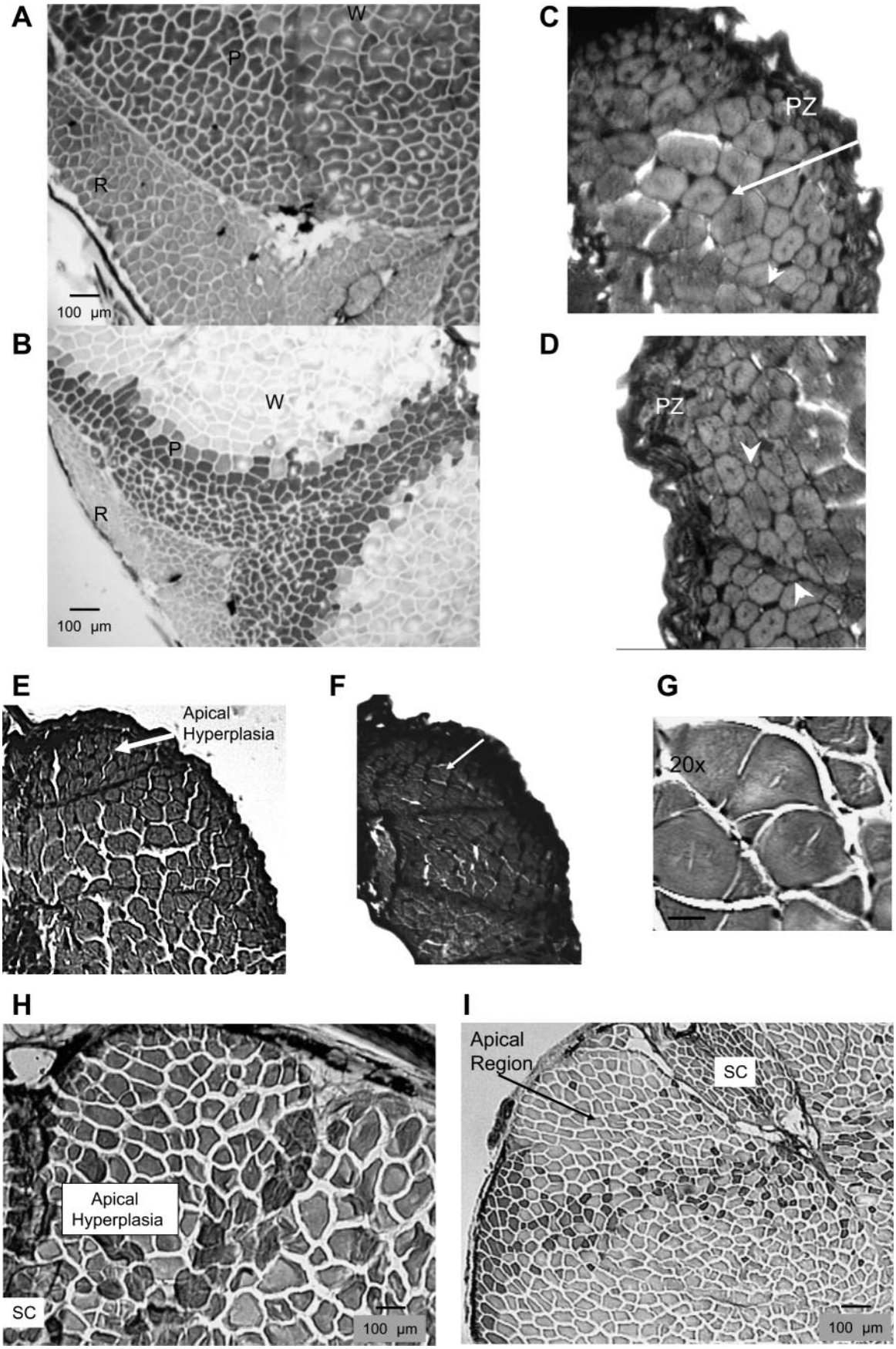
The histochemical analysis of myofibrillar ATPase activity showed differences between giant danio and zebrafish fiber-type lability (Fig. 7, *A* and *B*). However, consistent with most fish species, distinct fiber-type regions of the axial musculature (38) were detected in both species. Small differences were detected between the mATPase acid-stability of fiber types in giant danio (Fig. 7*A*). Giant danio pink muscle layer appears to be composed of fibers with strong acid-stable mATPase activity, while red and white fibers exhibit acid-stable mATPase activity at a lower level. In comparison, zebrafish exhibit a very distinct pink fiber layer with strong mATPase activity (Fig. 7*B*), similar to the guppy, rock goby, and goldfish (32). White fibers of the zebrafish exhibit low acid-stable mATPase activity, while red fibers exhibit moderate acid-stable mATPase activity. In both species, there is a gradual transition between pink and white muscle fiber layers, as pink fibers progressively become larger. In the zebrafish, these pink fibers also appear to lose their acid-stable mATPase activity during this progression as well. From these results, we can speculate that there might be an underlying effect of mATPase activity and muscle fiber recruitment in fish, as little difference was detected in ATPase lability in the rapidly growing giant danio. It is possible that there is a link between metabolic activity of muscle fibers and their ability to respond to endogenous cues, such as GH.

Growth trial. To further investigate the differential growth patterns between giant danio and zebrafish, a 17-wk growth trial was conducted to determine the effects of exogenous GH on overall growth in 1- and 2-yr-old giant danio compared with 2-yr-old zebrafish. GH is known to exhibit profound growth increases in several fish species (1, 22, 23). In the present study, overall growth increased ($P < 0.0001$) 46 and 64% over the entire 17-wk trial in both 2- (Fig. 8*A*) and 1-yr-old giant danio (Fig. 8*B*), respectively. At the termination of the study, the rbGH-treated 1-yr giant danios reached the size (4.57 $\pm$ 0.1 g) of the 2-yr control fish (4.43 $\pm$ 0.1 g). Interestingly, both age groups grew at a rate of 0.10 g/wk, suggesting that, regardless of beginning size, giant danios are capable of positively responding to a growth-promoting agent. In contrast, female zebrafish only exhibited an increased (Fig. 8*D*, $P < 0.0001$) weight following the first injection, while males showed no overall increase (Fig. 8*C*, $P = 0.07$) in growth. While female zebrafish exhibited an overall effect of rbGH ($P < 0.0001$), no treatment $\times$ time interaction was detected, suggesting that some of the effects observed could be due to ovarian cycling (i.e., egg production, release, and resorption). Visual observations of female zebrafish before and during the trial suggested that egg production appeared to occur in all females. These observations could explain the variation and fluctuations observed in overall weight in the female groups. Recent reports have demonstrated that rbGH treatment can increase gonad expression of both GH and IGF-I mRNA in trout (6). Sex-steroid hormones are also known to promote GH secretion and subsequent growth in fish species (9).

No difference in fiber number was detected in any group, regardless of age, species, or treatment (data not shown). rbGH treatment resulted in smaller mean fiber areas in 2-yr-old giant danio ($P < 0.05$; 1,710.57 $\pm$ 213.48 vs. 2,378.39 $\pm$ 207.78 μm^2 in rbGH and control fish; Fig. 9). Interestingly, in the 1-yr-old giant danio, no difference was detected in mean fiber area between treatment groups ($P = 0.303$; 1,621.123 $\pm$ 129.59 vs. 1,426.76 $\pm$ 127.63 μm^2 in rbGH and control fish). These results suggest that younger giant danio are more efficient at increasing growth via hypertrophic or a combination of hyperplastic and hypertrophic muscle growth, while older giant danio increase overall growth primarily through hypertrophic muscle growth. Consistent with the growth data, zebrafish adults exhibited no difference in mean fiber area or number in response to rbGH treatment (data not shown).

GH administration has been shown to promote hyperplastic growth in rainbow trout, *Oncorhynchus mykiss* (53), and Atlantic salmon, *Salmo salar* (13). GH transgenic coho salmon, *Oncorhynchus kisutch*, also demonstrate increased hyperplastic muscle growth compared with nontransgenic controls and same-age controls (24). Morphometric analysis of larval growth in giant danio and zebrafish demonstrated faster, more efficient growth in giant danio larvae. Consistent with this paradigm, adult giant danio respond to GH treatment continually, as 1- and 2-yr-old fish increased growth following rbGH administration over 17 wk. Similar to fast-growing trout and salmon, adult giant danio demonstrated hyperplastic muscle growth, suggesting that giant danio exhibit indeterminate growth similar to larger teleosts. Adult zebrafish did not exhibit increased growth or hyperplasia in response to rbGH, suggesting that zebrafish reach a growth plateau similar to mammals and hence exhibit determinate growth.

Collectively, the results of the larval growth study and the adult growth trial suggest that giant danios exhibit indetermi-



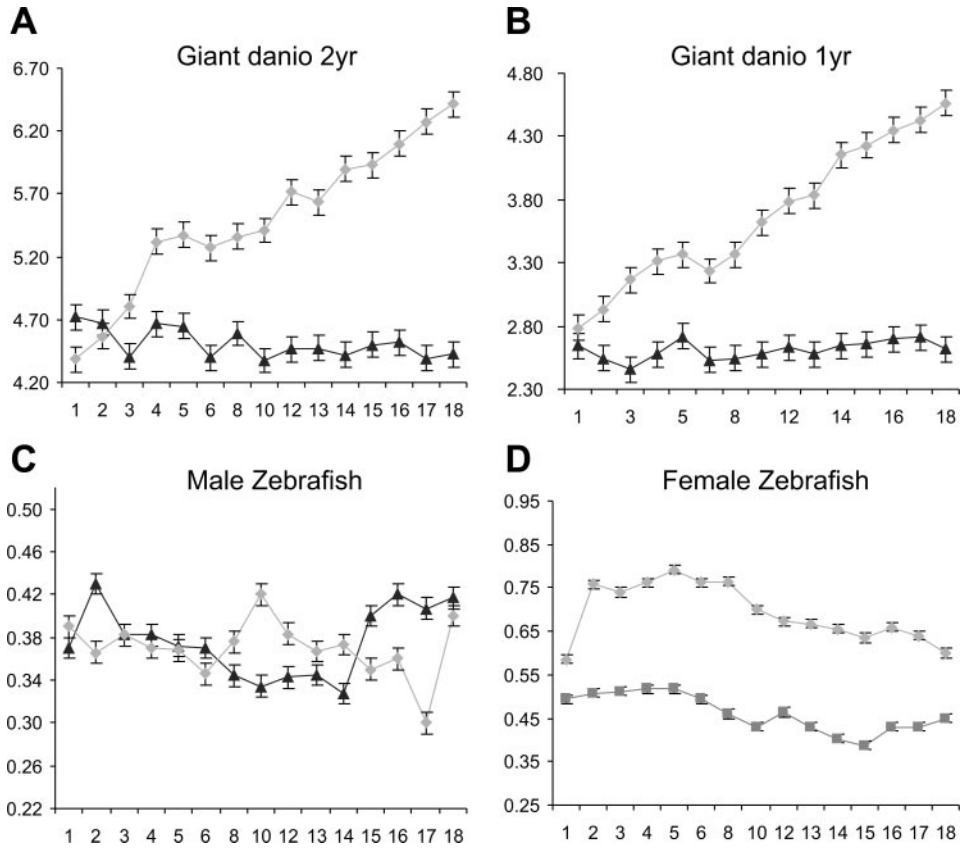


Fig. 8. Growth response (in grams) of giant danio (1 and 2 yr old) and zebrafish (2 yr old) to exogenous, sustained-release recombinant bovine growth hormone (rbGH). Solid line represents excipient control, shaded line represents rbGH treated in *A–D*. Weight in grams on *x*-axes and time in weeks on *y*-axes for *A–D*. rbGH increased 2-yr-old (*A*) and 1-yr-old (*B*) giant danio throughout the 17 wk, $P < 0.0001$. Male zebrafish (*C*) overall growth was not significantly affected by rbGH, $P = 0.07$, while female zebrafish (*D*) growth was increased overall by rbGH ($P < 0.0001$).

nate growth and maintain the ability to respond to a growth-promoting agent as adults, while zebrafish adults reach a growth plateau as they mature. This also suggests that the zebrafish and giant danio can be utilized as a direct comparative model system for muscle growth physiology studies, with the zebrafish serving as a model organism for mammalian-type growth and the giant danio serving as a model for growth in commercially important fish species like rainbow trout.

Fig. 7. Myofibrillar ATPase activity after pH 4.2 preincubation in adult fish. *A*: giant danio mATPase activity. Pink muscle (P) is composed of fibers showing more acid-stable mATPase activity than red (R) and white (W) fibers. *B*: zebrafish mATPase activity. Pink fibers show very acid-stable mATPase activity compared with red and white fibers. Scale = 100 μm. Magnification ×40. *C* and *D*: giant danio (3 wk old) epaxial myotome. *C*: the arrow represents the direction of fiber progression and growth. Arrowhead points to small-diameter fiber located within the presumptive white fiber layer, consistent with mosaic hyperplastic growth. Magnification ×500. *D*: proliferation zone (PZ) located beneath the superficial monolayer and the skin. Arrowheads indicate small-diameter fibers located within the presumptive white fiber layer. Magnification ×500. *E*: 4-wk-old giant danio larvae epaxial myotome, showing extensive small-diameter fibers within the apical region of the epaxial region. Magnification ×200. *F*: 4-wk-old zebrafish larvae epaxial myotome showing less apical small-diameter fibers. Magnification ×200. *G*: 1.5-yr-old giant danio epaxial myotome with small- and large-diameter muscle fibers resulting in characteristic mosaic appearance. Magnification ×20. Bar = 20 μm. *H*: 1.5-yr-old giant danio ventral epaxial myotome showing smaller diameter fibers within the apical region. Magnification ×40. *I*: 1.5-yr-old zebrafish ventral epaxial myotome showing no difference in fiber size in apical region. Magnification ×40. SC, spinal cord.

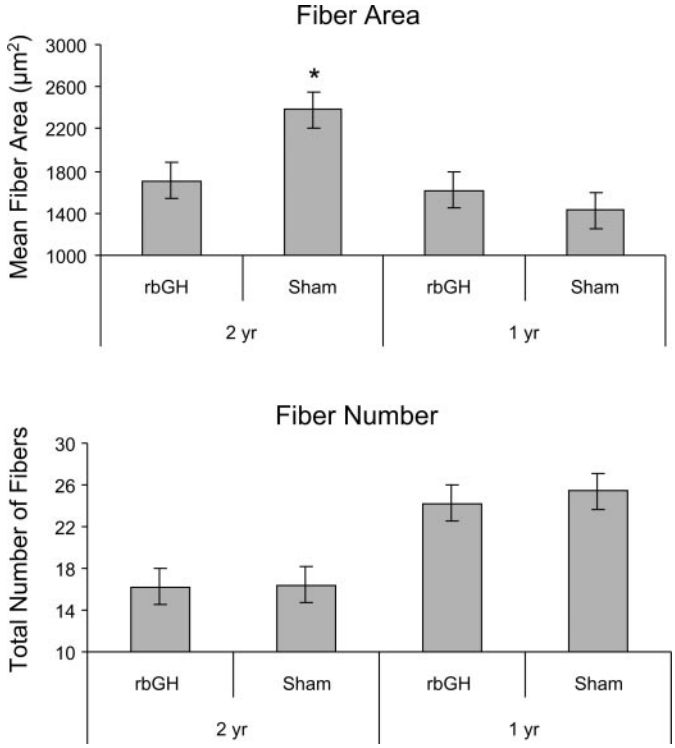


Fig. 9. A_f (μm²) and fiber number in 1- and 2-yr-old giant danio treated with rbGH or control. *$P < 0.05$.

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