Growth and Survival of Zebrafish (*Danio rerio*) Fed Different Commercial and Laboratory Diets

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Abstract

The need to develop standardized diets to support zebrafish (*Danio rerio*) research is supported by the knowledge that specific dietary ingredients, nutrients, or antinutritional factors in diets have been shown to affect development and growth of adult *D. rerio* and their offspring. In this study, there were seven dietary treatments consisting of five commercially available diets and two laboratory-prepared diets, three replicates per treatment. Fish were fed *ad libitum* twice daily for 9 weeks. At 9 weeks, both weight and length were recorded to determine condition indices. *D. rerio* fed one of the laboratory-prepared diets had significantly higher weights than individuals fed any of the other diets and exhibited significantly higher lengths than those fed five of the six remaining diets. Although there were significant differences in general growth demographics (length/weight) after the 9-week feeding trial, no significant differences in overall health of *D. rerio* were observed for the different dietary treatments as determined by statistical analysis of condition factor indices ($K = [\text{weight} \times 100] / \text{length}^3$). The success achieved with the laboratory-prepared diets represents the foundation for establishing an open-formulation nutritional standard to ensure that the *D. rerio* model for research does not generate confounding research results caused by nutritional vagaries.

Introduction

ZEBRAFISH (*DANIO RERIO*) are an important laboratory model to study development, genetics, and human disease, as they undergo rapid development, display genetic similarities to humans,¹ and can develop many disease pathologies² by induction. Of major concern, however, is the lack of nutritional control due to the absence of a standardized reference diet.³ Many ingredients that are common components of aquatic fish diets contain compounds that can alter the physiology and behavior of the organism.^{4,5} Of recent interest are soy isoflavones, commonly found in aquatic flake foods, which have been implicated as biocontaminants (estrogen mimics), and may compromise the interpretation of experimental results.^{6–8} Epidemiological studies of several mammalian species suggest that prenatal and perinatal diets are important determinants of the health of an organism.^{9–12} Markovich *et al.*¹³ observed that *D. rerio* fed a flake diet produced significantly fewer eggs than those fed other diets,

while Jaya-Ram *et al.*¹⁴ demonstrated that the level of dietary highly unsaturated fatty acid affected *D. rerio* egg production and hatchability. These results suggest that diet affects *D. rerio* as similarly observed in mammals and support the need to establish and use a dietary standard.

Within the existing variability of *D. rerio* feeding protocols, few studies have evaluated the effect of diet on adult *D. rerio* growth and survival. Meinelt *et al.*^{15,16} determined that growth and fertilization rates of adult *D. rerio* were affected by the dietary level of n-6 polyunsaturated fatty acids, indicating that the presence or absence of specific nutrients can affect normal physiological processes in *D. rerio*. Our objective was to assess the overall growth responses among *D. rerio* fed different commercially available feeds that have been commonly used to maintain adult *D. rerio* for experimentation. In addition, we report the ingredient content and comparative efficacy of two laboratory-prepared diets formulated and tested to serve as the prototype diets toward realizing the ultimate goal of establishing an open-formulation nutritional standard.

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Materials and Methods

Experimental system

The experimental system consisted of 21 rectangular tanks (35 L volume) connected to a semiclosed (replaced with 20% new filtered water weekly [375 μ S, 125 mg/L CaCO_3]; Seagull IV; General Ecology, Exton, PA) 1164-L indoor recirculating system (Mars Marineland Retailer Systems; Spectrum Brands, Atlanta, GA). Freshwater was pumped from the sump through two ultraviolet sterilizers (AquaFine, Valencia, CA) at a recirculating rate of 5.4 L/min/tank (11,108% daily exchange/tank/day). Mechanical filtration was accomplished using woven floss, while biological filtration was achieved using 82 L of 0.04-m-diameter bioballs media. A light:dark photoperiod of 12:12 h was provided by indirect fluorescent lighting. Temperature was monitored daily using a thermometer (Fisher Scientific, Pittsburgh, PA). Ammonia-nitrogen, NO_2 -N, and pH were monitored weekly using an aquarium pharmaceuticals test kit (Aquarium Pharmaceuticals, Chalfon, PA). Each tank contained one cylindrical plastic mesh cage (0.25 m height by 0.09 m diameter) covered with nylon mesh (Fashion Knee Hi; American Corporation, Henderson, NC). Temperature was maintained at 28°C ($\pm 0.5^\circ\text{C}$), pH ranged between 8.0 and 8.4, and ammonia-nitrogen and NO_2 -N remained below visual detection limits.

Fish

Wild-type zebrafish obtained from a local pet store were spawned as described by Westerfield¹⁷ and reared 28 days postfertilization on rotifers (*Brachionus plicatilis* ["L" type]) fed an enriched diet (Reed Mariculture, Campbell, CA). To minimize handling stress, these 28 days postfertilization fish were individually photographed in reduced-volume glass containers with reference grids, and lengths (mouth to caudal peduncle) were determined by image analysis (Image-J; National Institutes of Health, Bethesda, MD). Fish were then randomly assigned (using Microsoft Excel's random number generator) to individual mesh cages (10 fish per mesh cage, three mesh cages per diet) and twice daily fed *ad libitum* (all diets fed to visible excess ~5 min after feeding; the physical characteristics of the feed did not affect feeding) one of five commercially available diets or two laboratory-prepared diets. All experimental fish were photographed and measured for length at the end of 3-week intervals for the duration of the 9-week experiment. At the conclusion of the experiment, both weight (mg) and length (mm) were determined to calculate condition factor indices ($K = [\text{weight} \times 100] / \text{length}^3$).¹⁸

Diets

Dietary treatments that were evaluated consisted of five commercially available and two laboratory-prepared diets. The five commercial diets that served as dietary treatments were as follows: Aquamax Grower 400® (45% protein, 16% fat, and 3% fiber; PMI Nutritional International, LLC., Brentwood, MO; Lot # 5D04), Cyclop-eeze® (60% protein, 31% fat, and 0.5% fiber; Argent Laboratories, Redmond, WA; Lot # 204597), Nutrafin Max Flake Food® (44% protein, 5% fat, and 2% fiber; Rolf C. Hagen Corporation, Mansfield, MA; Lot # 60401), TetraMin Tropical Flakes® (48% protein, 8% fat, and 2% fiber; Tetra Holding, Blacksburg, VA; Lot # 17B29), and Tetra Pond Koi Vibrance® (31% protein, 5% fat, and 2% fiber;

Tetra Holding; Lot # 09E28). The remaining two laboratory-prepared diets were adopted from Kovalenko *et al.*¹⁹ Diet A was formulated to contain 45.4% protein and 33.2% lipid, while diet B contained 41.4% protein and 33.2% lipid. Proximate composition was provided by respective manufacturers or analytical analysis (Mississippi State Chemical Laboratory, Mississippi State, MS) for the commercial and experimental diets, respectively.

Preparation of laboratory diets

The ingredient compositions of the laboratory-prepared diets tested are presented in Table 1. Casein, canthaxanthin, fish protein hydrolysate, rice starch, soy lecithin, and wheat gluten were added to a beaker containing distilled water (200 mL/100 g of diet) and mixed using a stir bar. Menhaden oil, cholesterol, ascorbylpalmitate, a vitamin premix, betaine, choline chloride, a mineral premix, monopotassium phosphate, and glucosamine were then added and mixed. Egg yolk or defatted egg yolk (prepared according to Chung and Ferrier²⁰) was then mixed with the other ingredients and homogenized (in a VirTishear homogenizer [The Virtis, Gardiner, NY] for 3 min at 2000 rpm). The required amount of alginate was then added, followed by additional homogenization for 2 min at 2000 rpm. The resulting thick paste was then autoclaved at 554 g/cm³ for 20 min. The paste was allowed to cool to room temperature, spread on plastic wrapped trays, and fan-dried for 48 h. The resulting dry sheets of feed were added to a coffee bean grinder (Black and Decker; Applia Consumer Products, Miramar, FL) and ground to achieve a particle size that passed through a 1-mm² mesh. Feeds were then stored at 4°C until used.

Statistical analysis

Length, weight, and condition index values were statistically compared using analysis of variance (ANOVA) (v12.0; SPSS, Chicago, IL) to determine if significant differences existed among the diets. If significance was indicated, then significant differences ($p < 0.05$) between individual treatments were determined using the Student-Newman-Keuls inequality. Differences in length, weight, and condition index between sexes within diets were statistically compared using the independent-samples *T*-test and then compared by sex across diets using ANOVA. Tests for normality and constant variance were performed before all statistical analysis.

Results

No differences in feeding behavior were observed among the different dietary treatments throughout the 9-week study. All feeds utilized in the trial initially floated and were readily consumed, but would sink over time. Zebrafish continued consuming feed particles on the bottom of the enclosed fine-mesh cages. At the end of the experiment, zebrafish fed laboratory-prepared diet A gained significantly more weight (237.1 mg) than those fed the other diets (Table 2). Mean weights for zebrafish fed the other diets ranged between 70.6 mg (Cyclop-eeze) and 178.5 mg (TetraMin Tropical Flakes). Mean length of zebrafish fed laboratory-prepared diet A (26.4 mm) was significantly longer than that of those from each of the other dietary treatments, except laboratory-prepared diet B (24.2 mm; Table 2). Although length and weight were sig-

TABLE 1. COMPOSITION OF THE LABORATORY-PREPARED A AND B DIET

Ingredient ^a	Inclusion level (g 100/g)		Ingredient	Inclusion level (g 100/g)	
	A	B		A	B
Ascorbylpalmitate ^b	0.04	0.04	Menhaden oil ^b	5.63	0.00
Betaine ^b	0.15	0.15	Menhaden:corn 2:1 ^b	0.00	18.00
Canthaxanthin (10%) ^c	2.31	2.31	Mineral premix ^{d,e}	1.54	1.54
Casein ^d	14.70	14.70	Potassium phosphate ^b	1.15	1.15
Cholesterol ^b	0.12	0.12	Refined soy lecithin ^f	1.90	2.90
Choline ^b	0.38	0.38	Rice starch ^b	7.70	12.80
Egg yolk ^b	38.45	0.00	Sodium alginate ^g	5.38	5.38
Partially defatted egg yolk ^b	0.00	19.98	Vitamin premix ^{d,h}	1.15	1.15
Fish protein hydrolysate ⁱ	15.40	15.40	Wheat gluten ^b	3.85	3.85
Glucosamine ^b	0.15	0.15			
Ash (g/kg)	5.9	5.5	Energy (kcal/kg)	586	591
Dry matter (g/kg)	83.2	81.6	Crude protein (g/kg)	45.4	41.4
Crude lipid (g/kg)	33.2	33.2	PE ratio (g/cal)	77.4	70.0

^aLot numbers of ingredients utilized in experimental diets: ascorbylpalmitate (19F3441), betaine (29H0717), canthaxanthin (UT99075290), casein (5160E), cholesterol (77H0499), choline (7620), egg yolk (E-0625), glucosamine (20K0958), menhaden oil (90K0888), mineral premix (4710), potassium phosphate (5379), refined soy lecithin (108856), rice starch (25H0267), vitamin premix (2935), and wheat gluten (100K0191).

^bSigma-Aldrich (St. Louis, MO).

^cDSM Nutritional Products (Belvidere, NJ).

^dMP Biomedicals, LLC (Solon, OH).

^eComposition of the mineral premix (%): calcium carbonate, 2.100; calcium phosphate dibasic, 73.500; citric acid, 0.227; cupric citrate, 0.046; ferric citrate, 0.558; magnesium oxide, 2.500; magnesium citrate, 0.835; potassium iodide, 0.001; potassium phosphate dibasic, 8.100; potassium sulfate, 6.800; sodium chloride, 3.060; sodium phosphate, 2.140; zinc citrate, 0.133.

^fUSB Corporation (Cleveland, OH).

^gISP Alginates (San Diego, CA).

^hComposition of the vitamin premix (%): ascorbic acid, 12.5; BHA, 0.1; biotin, 0.1; cellulose, 60.0; calcium pantothenate, 1.5; cobalamin, 0.1; folic acid, 0.5; inositol, 18.0; nicotinic acid, 2.6; PABA, 3.0; pyridoxine HCL, 0.3; riboflavin, 0.8; thiamine mononitrate, 0.5.

ⁱThe Scoular Company (Minneapolis, MN).

nificantly different among dietary treatments, overall condition (as assessed through condition index) did not differ significantly among the treatments (Table 2). Zebrafish fed Tetra Pond Koi Vibrance had the lowest mean value for length and second lowest for mean weight; however, survival for this dietary treatment was 100% (Table 2). Mean survival for the other dietary treatments ranged between 67% (TetraMin Tropical Flakes) and 80% (laboratory-prepared diet A, Cyclop-eeze, and Aquamax Grower 400). No significant differences were determined for weight, length, and condition factor between male and female zebrafish within an individual diet (Table 3). Mean weights for male zebrafish ranged from 71.6 mg (Tetra Pond Koi Vibrance) to 180.9 mg (laboratory-prepared diet A), and from 68.0 mg (Cyclop-eeze) to 279.2 mg (laboratory-prepared diet B) for female zebrafish. Mean lengths ranged from 18.7 to 25.5 mm (Tetra Pond Koi Vibrance and laboratory-prepared

diet A, respectively) and 17.8 to 27.1 mm (Tetra Pond Koi Vibrance and laboratory-prepared diet A, respectively) for male and female zebrafish, respectively (Table 3). Condition factor was not significantly different for male or female zebrafish fed the different diets.

Discussion

The current investigation evaluated the relative effectiveness of commercially available diets that have been fed in zebrafish culture in the past versus laboratory-prepared diets. These data provide a foundation for the future development of defined, open-formulation diets with a proposal for future dietary standardization. While future studies should focus on diets that enhance breeding by comparing the cross success rate, this investigation and corresponding results presented

TABLE 2. MEAN GROWTH, SURVIVAL, AND CONDITION INDICES ($\pm$ SEM) OF *DANIO RERIO* FED ONE OF FIVE COMMERCIAL OR TWO LABORATORY-PREPARED DIETS *AD LIBITUM* AT THE END OF A 9-WEEK GROWTH TRIAL

Diet	Survival (%)	Length (mm)	Weight (mg)	Condition factor (K)
Aquamax Grower 400 [®]	80.0	22.6 (1.0) ^{b,c}	158.1 (21.4) ^b	1.19 (0.05) ^a
Cyclop-eeze [®]	80.0	19.4 (1.2) ^a	70.6 (10.8) ^a	0.91 (0.09) ^a
Experimental diet A	80.0	26.4 (1.0) ^d	237.1 (29.3) ^c	1.22 (0.14) ^a
Experimental diet B	76.7	24.3 (0.9) ^{c,d}	168.9 (18.2) ^b	1.07 (0.06) ^a
Nutrafin Max Flake Food [®]	73.3	19.9 (0.8) ^{a,b}	119.0 (18.4) ^{a,b}	1.43 (0.30) ^a
TetraMin Tropical Flakes [®]	66.7	23.2 (0.8) ^c	178.5 (21.1) ^b	1.33 (0.07) ^a
Tetra Pond Koi Vibrance [®]	100	18.3 (0.8) ^a	75.6 (12.7) ^a	1.08 (0.14) ^a

Means with different superscript letter designations within columns are significantly different ($p < 0.05$).

TABLE 3. MEAN GROWTH AND CONDITION INDICES ($\pm$ SEM) OF MALE AND FEMALE *D. rerio* FED ONE OF FIVE COMMERCIAL OR TWO LABORATORY-PREPARED DIETS *AD LIBITUM* AT THE END OF A 9-WEEK GROWTH TRIAL

Diet	Sex	Length (mm)	Weight (mg)	Condition factor (K)
Aquamax Grower 400®	M (9)	21.4 (1.3) ^{a,b}	130.1 (29.4) ^{a,b}	1.14 (0.08) ^a
	F (11)	23.6 (1.4) ^{b,c}	180.9 (29.9) ^{a,b}	1.23 (0.05) ^a
Cyclop-eeze®	M (12)	19.1 (1.5) ^a	73.0 (16.4) ^a	0.89 (0.13) ^a
	F (11)	18.0 (1.4) ^a	68.0 (14.7) ^a	0.92 (0.14) ^a
Experimental Diet A	M (9)	25.5 (1.7) ^b	180.9 (27.8) ^b	1.02 (0.07) ^a
	F (12)	27.1 (1.2) ^c	279.2 (44.0) ^b	1.38 (0.23) ^a
Experimental Diet B	M (12)	24.6 (0.9) ^b	159.7 (24.7) ^{a,b}	0.98 (0.10) ^a
	F (11)	23.9 (1.6) ^{b,c}	179.0 (27.9) ^{a,b}	1.18 (0.05) ^a
Nutrafin Max Flake Food®	M (11)	19.4 (1.1) ^a	86.6 (15.4) ^a	1.07 (0.08) ^a
	F (11)	20.5 (1.3) ^{a,b}	151.5 (31.2) ^a	1.79 (0.58) ^a
TetraMin Tropical Flakes®	M (12)	23.1 (1.0) ^{a,b}	171.3 (24.8) ^b	1.32 (0.11) ^a
	F (6)	23.6 (1.5) ^{b,c}	193.0 (42.0) ^{a,b}	1.35 (0.07) ^a
Tetra Pond Koi Vibrance®	M (15)	18.7 (1.2) ^a	71.6 (17.8) ^a	0.98 (0.15) ^a
	F (13)	17.8 (0.9) ^a	80.2 (18.9) ^a	1.21 (0.26) ^a

Means with different superscript letter designations within columns are significantly different from those within the same sex across diets ($p < 0.05$). No significant differences were found for weight, length, and condition factor between male and female zebrafish within an individual diet. M, male (n); F, female (n).

clearly demonstrate to the zebrafish community the importance of feeding defined diets and the potential experimental problems that may arise from utilizing undefined diets.

Published concentrations of major dietary constituents vary significantly for commercial feeds used to culture *D. rerio* that are bred to be used specifically for research. Among diets tested in this study, crude protein ranged from 31% to 60% and crude lipid from 5% to 34%. Minerals and vitamins, feed ingredient sources, and trace elements also varied. These differences may be responsible for the observed differences in growth rates among individuals fed the different commercially available diets and those fed the laboratory-prepared diets. Although growth rates themselves are not necessarily indicative of health, they do suggest that *D. rerio* does respond to the quantity and/or quality of specific nutrients required for normal rates of growth. Additionally, the diets may influence other physiological processes, including those associated with endocrine, neurological, immunological, or reproductive function. These and other dietary-mediated processes may have contributed to observed differences in survival. The lack of significant differences in condition index, which is historically considered a good indicator of health in fish, suggests that the fish were compositionally similar when adjusted for length "as a decline in condition factor is usually interpreted as depletion of energy reserves such as stored liver glycogen or body fat."²¹ Condition indices have not been used in zebrafish; consequently, condition indices based on basic size demographics may be of limited value in addressing localized compositional changes in specific tissues or organs. Further, differences in sexual maturity were visually observed, suggesting that certain still undefined dietary nutrients are necessary to produce sexually mature zebrafish at 13 weeks, further implying the importance of a nutritionally complete *D. rerio* diet.

In this study, the two semipurified diets prepared and tested represent a first attempt at developing semipurified diets with ingredients that are chemically well defined. These diets were similar in nutrient profiles, but differed primarily in ingredient composition, particularly the sources of energy (lipid and carbohydrate). Although these diets were isocaloric

and contained similar levels of macronutrients, weight gain of individuals fed diet A was significantly higher than that of diet B, suggesting that the source of specific nutrients affects those physiological, cellular, or molecular processes that contribute to weight gain.

Although many researchers utilize natural diets (mainly *Artemia* sp.), development of research-grade *D. rerio* diets is still vitally important as the nutritional quality of *Artemia* varies considerably with geographical strain,^{22,23} rearing conditions,²⁴ and their nutritional history.^{25,26} This high degree of nutritional variability may affect growth and other physiological parameters over the course of a feeding trial, making direct comparison to defined dry feeds difficult. This, however, does not negate the importance of designing experimental feeds with nutritional profiles similar to those found in cultured *Artemia*. The nutritional profile of Great Salt Lake brine shrimp (*Artemia franciscana*) cultured in a defined unialgal system contains $9.2 \pm 0.8\%$ dry matter, with 77.4% of this dry matter composed of ash ($13.5 \pm 0.5\%$), carbohydrate ($3.6 \pm 0.3\%$), lipid ($9.5 \pm 0.7\%$), and protein ($50.8 \pm 0.2\%$).²² With a few exceptions, this nutritional profile compares favorably with those from the seven dietary treatments utilized in this study.

Application of zebrafish research in the last 15 years has led to the development of purported paradigms that suggest similarities in the genetics of human and *D. rerio* disease. Unfortunately, many of the same mistakes that were committed historically in disease research using rodent models may be repeated with the zebrafish model. Numerous studies have shown that specific nutrients, or specific compounds found in feed ingredients, can substantially affect the outcomes of experiments.²⁷ In a recent study conducted in our lab, zebrafish fed a ration containing the isoflavone genistein accumulated genistein in the tissue at levels proportional to the feed content (Siccardi III, unpublished data). These physiologically relevant concentrations of genistein could potentially affect research outcomes in which estrogen mimics have consequence. Recent animal studies have also shown how differences in dietary arsenic, commonly found in trace amounts in some ingredients, profoundly altered gene expression and confounded genomic analysis in mouse liver

and lung.²⁸ These studies clearly demonstrate the effect that diet can have on interpreting experimental results both within and between laboratories. Nutrition is now known to be an important determinant of disease progression in all rodent models, and authors are now obliged to report the specific diet used in those ongoing investigations. Nutrition and its consequences have yet to be included in zebrafish research endeavors. In the absence of an understanding and standardization of dietary intake, the results of ongoing studies are subject to varied interpretation as influenced by the physiology of adult *D. rerio* and the subsequent development of offspring derived from these adult populations.

Zebrafish researchers must come to an accord in the development and common use of an open-formulation dietary standard. Feeds that are currently used contain different concentrations of a variety of compounds known to produce confounding variation in results of molecular experimentation with other animal models. Variation in commercial rodent diets even within brands has been shown to influence experimental results.²⁹ This variability is difficult to manage between rodent studies due to the yearly volume of feed produced; however, the reduced feed utilization by zebrafish researchers could allow commercial feed companies to produce a single batch of feed that could be utilized by all members of the zebrafish community over a defined period of time. Until standardized *D. rerio* diets are established and ultimately utilized by the zebrafish community, confidence in the results among different laboratories that utilize different dietary regimes cannot be fully realized.

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