Contested Paradigm in Raising Zebrafish (*Danio rerio*)

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Abstract

The current body of work on rearing larval/juvenile zebrafish is based on (1) utilization of freshwater and (2) diurnal light/dark cycle, (3) provision of live feed at modest density, and (4) culture in high visibility environment. We challenged these rearing approaches by maintaining zebrafish under constant light for 46–48 days (days post-fertilization [dpf]), while securing continuous feeding in high turbidity and saline (1.8–2.1 parts per thousand) environment for the experiment's duration, allowing 24 h feeding/growth of fish from first exogenous feeding to maturation. There was no evidence of negative effects on zebrafish larvae behavior, growth, survival, and life cycle duration at constant illumination when food was continuously available. Zebrafish were stocked at high initial density (100 larvae/L) in a static system and fed high densities of rotifers (*Brachionus plicatilis*) (200–400/mL) from 6 to 12 dpf. Fish density was then reduced by 50% and two diet treatments, live rotifers or brine shrimp (*Artemia*) nauplii (10/mL), followed. Fish were reared on these two diets until first maturation. Performance of adult zebrafish fed live rotifer followed by *Artemia* nauplii diet was the highest recorded in the literature after 42 dpf, 250 ± 29 (males) and 430 ± 5 mg (females). Use of these rearing conditions, during the entire life cycle, until reproduction, resulted in the shortest ever recorded generation time (from egg to egg) of 43–45 dpf and fertilization rate (1 dpf) of 80.3%–94%.

Keywords: fish breeding, brine shrimp, larval feeds, fish growth, zebrafish generation time

Introduction

THE MAJOR DRIVING PRINCIPLES of this work were to achieve maximum potential performance of zebrafish, maintain high survival (health), minimize deviation in growth among individual fish, and obtain sex-associated uniform size at the time of maturation. Streisinger *et al.*¹ emphasized the importance of the nutritional regime and stressed dietary supplements for parental fish, in particular, to obtain homozygous fish, naturally more susceptible to nutritional deficiencies. However, larval/juvenile zebrafish feeding procedures have changed very little over the last 30 years and continue to include protozoa (*Paramecium* sp.) as initial diet followed by brine shrimp nauplii (salt water organism). Therefore, to advance the success of larval/juvenile zebrafish rearing in laboratory conditions, this study reevaluated the natural habitat of this species^{2,3} and took into account both abiotic indicators and biological characteristics of zebrafish. This study also adapted the wealth of knowledge on optimization of larval fish growth in intensive culture systems of commercial fish species to the physiology and behavior of zebrafish.

Current zebrafish rearing procedures are almost uniformly based on diurnal light/dark cycle (14:10 h), provision of live feed at modest density, and high visibility environment.^{4–9}

Consequently, several authors have suggested standardized conditions for larval zebrafish rearing; however, there remains a discrepancy in fish growth and a great variation in fish size at age despite this standardization. For instance, Von Hertel *et al.*¹⁰ reported that the coefficient of variation in zebrafish weight after 4 and 12 weeks of growth was 55% and 60% of the mean, respectively. Parichy *et al.*⁷ have shown that individual variation in 49 days postfertilization (dpf)-old zebrafish growth trajectories amounted to between 9 and 12.8 mm, and the deviation in size continued to increase. Gomez-Requeni *et al.*⁸ raised zebrafish at 28°C and obtained a growth curve that at 16 and 30 dpf resulted in 6.84 and 16 mm, respectively. The authors claimed that these growth rates established “baseline data for further nutritional studies”; however, Barrionuevo *et al.*⁹ obtained only 6.3 mm fish after 30 dpf in normoxic conditions at 28°C, which is below this suggested “baseline.” Therefore, no “standards” of acceptable growth rate exist in the literature, although it is frequently extremely slow (0.057–0.14 mm/day).⁷

Magnuson¹¹ has argued in the past when working with Japanese medaka (*Oryzias latipes*), that high food availability (excessive zooplankton density) would result in elimination of variability in growth within a population. However, excessive and continuous availability of food to zebrafish

larvae have not been examined. Therefore, we reexamine this question and offer a new approach, continuous, 24-h availability of food to zebrafish experimental units, which may contribute to the elimination of excessive deviation in fish size within a population.

It is well documented that fish size (length and weight) must be used as criteria to accurately express morphological development during transformation from the larval to juvenile phase, frequently referred to as metamorphosis, as organs and their function “appear and disappear” in early ontogeny.¹² More noticeable are the distinct ontogenetic changes in external appearance of fish. For example, eye migration in flatfish,¹³ as well, and morphological and functional differences in the gills of early ontogeny cyprinids¹⁴ and percids¹⁵ involving respiratory, excretory, and osmoregulatory functions.¹⁶ Rombough¹⁶ argued that osmoregulation preceded respiratory role as the major function of gills in zebrafish ontogeny. Therefore, using fish size (length) at which particular developmental stages occur provides the only accurate description of the ontogenetic event rather than age.¹⁷ In other words, it is far more accurate to express “smaller variance in the length at which selected developmental stages are reached rather than at the age at which they occur.”¹⁸

Balon¹⁹ demonstrated that larval stage with characteristic fin folds may persist in cyprinid larvae, *Blicca bjorkna*, for 60 days when fed at maintenance ration, in comparison to conditions of sufficient food availability, when the same stage of postmetamorphosis can be reached perhaps in 6 days. This is particularly critical in respect to the nearly exclusive use of zebrafish age in description of sequence of ontogenetic events such as gametogenesis and sex differentiation at present.²⁰

There has been numerous works on the effects of different light regimes on zebrafish biology. We challenged the rearing conditions almost uniformly used by investigators to grow zebrafish during larval/juvenile transition and for the first time maintained zebrafish at constant light for 46–48 dpf. By combining constant illumination with continuous live food availability, we secured 24-h feeding/growth of fish for the duration of the experiment. Villamizar *et al.*²¹ found that zebrafish do not survive in continuous darkness, and Zhdanova²² summarized data suggesting that zebrafish present behavioral and physiological characteristics similar to those of mammalian sleep during both light and dark, the frequency of which is light dependent.²³ When tench (*Tinca tinca*), another cyprinid species, of larval size similar (4.5 mm) to zebrafish, were fed live *Artemia* nauplii under continuous light (24:0) or under 12:12 light:dark regime over 20 days, fish reached final mean weights of 88 and 32 mg, respectively.²⁴ Similarly, European perch (*Perca fluviatilis*) larvae fed under continuous light in comparison to short day, had significantly higher survival, grew faster (68 and 21 mg, respectively), and had less heterogeneous size.²⁵

However, there is rather weak evidence of the negative effect that continuous light has on zebrafish growth and life cycle, although zebrafish has been used as the model for ontogenetic changes in sleep cycles as observed in humans.²⁶ For instance, Villamizar *et al.*²¹ argued that 7 dpf larvae reared under constant light had “significantly” smaller size than fish reared in conditions of light–dark cycle. However, mean size of these fish was 4 and 4.1 mm, respectively, suggesting generally negligible growth when kept under these regimes. The fish fed from 7 to 32 dpf under constant light reached 6 mm, suggesting

extremely unfavorable food conditions. There is, however, an agreement that zebrafish larvae cannot feed in the dark, even on prey as small and slow as *Paramecium*,²⁷ although Carrillo and McHenry²⁸ argued to the contrary. The latter authors performed experiments where zebrafish larvae initially fed with rotifers (5–10 dpf) were then forced into dark conditions in 3 mm deep containers and offered *Artemia* nauplii. Some incidental ingestion of *Artemia* was observed. However, the total mortality of zebrafish larvae in the dark was recorded at 20 dpf.²¹ Some fish species require complete dark during larval rearing (South American catfish, *Pseudoplatystoma* sp.), as light conditions result in severe cannibalism.²⁹ Mukai and Lim³⁰ documented that high survival of larvae of Asian catfish (*Pangasionodon* sp.) in dim light (60%) was three times higher than in the dark or high light intensity conditions.

Survival and growth of fish larvae in turbid water conditions, either caused by algae or inorganic clay, are significantly improved as commonly practiced with larvae of marine and freshwater fish. In comparison to clear water, turbidity increases zooplanktonic organisms’ contrast, decreases prey reaction distance, results in dispersed light conditions, and consequently, frequently leads to dramatic decrease of aggressive interactions among larvae, clinging behavior, and cannibalism (see well-described phenomenon in percid fishes).³¹ Larval zebrafish are frequently exposed to high turbidity (“green water”) conditions in their natural environment,^{2,32} and the highest density of zebrafish was recorded in areas of lowest Secchi depth (15 cm) (lowest visibility). Therefore, it was natural to explore the effect of turbidity on zebrafish larvae and juvenile performance.

Algae-induced turbidity has positive impact on food (rotifer) acceptance and larval survival and growth,^{33–35} and this effect can be associated with improving nutritional and palatability value of rotifers, increased visual acuity (contrast), decreased aggressive interactions and stress among larvae maintained at high density, removal of toxic metabolites from rearing water, and inhibition of growth of potential bacterial pathogens.³⁶ The net result is stabilization of the rearing system for fish larvae and decreased risk of accidental infection, or deterioration of the quality of the rearing environment. Szyper³⁷ documented that energy value of brine shrimp nauplii and rotifers (*Brachionus* sp.) starved for 23 and 48 h decreased 22.3% and 61.0%, respectively. Therefore, enrichment of zooplanktonic organisms with algal suspension, while simultaneously providing turbidity, is one of the principles of the described new rearing protocol.

There have been numerous studies on the effects of salinity on zebrafish during early life³⁸ and in adults.³⁹ Zebrafish embryos exposed to 6–8 parts per thousand (ppt) salinity from gastrulation to hatching survived at 100% and survival decreased at 10 ppt at 27°C.⁴⁰ De Marco *et al.*⁴¹ demonstrated the linear relationship between the whole-body cortisol of zebrafish larvae and the range of sodium chloride concentration up to 5.84 ppt. However, zebrafish behavior recorded as difference in space use, equivalent of consumed *Paramecium*, increased when larvae were preexposed in 50 mM (2.94 ppt) salinity. This result contradicts findings by Rothen *et al.*³⁸ that zebrafish yolk sac or free-swimming larvae suffered 50% and 30% mortality after 48 h of exposure to sodium chloride solution of 3 ppt.

Our intent was to use the salinity that would simultaneously provide high survival of zebrafish larvae and its live prey, saline water organisms, *Brachionus* and *Artemia*. Conte

*et al.*⁴² observed that 0.1 M (5.9 g/L) sodium chloride secured 100% survival of brine shrimp nauplii, while at lower salinity (0.05 M), survival is over 50%. In parallel, Minkoff *et al.*⁴³ reported that certain geographical strains of *Brachionus plicatilis* achieved the highest reproductive rate (exponential growth) at salinity of 4 ppt, and then the rate linearly decreased and reached no-growth level at 48 ppt. Therefore, there is ample evidence that there is a range of salinity that will support both growth of zebrafish larvae and its saline water live prey.

Zebrafish, compared to other vertebrates, provide many advantages for research aimed at genetic, embryonic, physiological, cellular, and molecular aspects of development and ontogeny. However, as Nusslein-Volhard *et al.*⁴⁴ concluded, “the generation time of 2–4 months, is not particularly short.” Shortening the generation time (from egg to egg) of zebrafish considerably, compared to earlier rearing results of 90 dpf⁴⁵ to 60 dpf,⁴⁶ would greatly benefit the research community. By using freshwater rotifers as the initial food, Aoyama *et al.*⁵ were able to obtain the first embryos after 46 dpf and consistent reproduction at 50 dpf. Therefore, improving growth at early life stages, when daily growth rate is ~15.8%/day at size 5–50 mg and later, at 50–150 mg, when growth rate decreases to 4.7%/day,⁸ should significantly shorten generation time. This would allow more multigenerational studies, compared to recent work by Shinya and Sakai⁴⁷ where 15 generations took ~48 months of studies.

Materials and Methods

Fish and breeding

Zebrafish of AB/TL hybrid line (Beattie, C., Department of Neuroscience, Ohio State University, Columbus) served as the experimental broodstock (10 females and 18 males; 6–12 months old). Fish were set in handmade breeding boxes fitted with perforated bottoms. Males and females were separated by plastic mesh, set in 37 L aquaria, and left in the dark overnight (14 h). The following morning, the plastic mesh barrier was removed and zebrafish were allowed to spawn naturally for 2 h. Resulting embryos were removed from the aquaria, combined, and placed in egg baskets for incubation within a zebrafish recirculation system (28°C) throughout embryonic development and hatching (3 dpf).

Experimental system

The rearing trials were carried out in a stagnant water system consisting of round 8 L polypropylene poly round

(PR) containers (25.2 cm diameter × 27.4 cm height) set in a water bath to maintain temperature (27–29.5°C) (Fig. 1). Each container held 6 L saline water of 1.8–2.1 ppt and was fitted with aeration. Live feed, rotifers, *B. plicatilis*, followed by brine shrimp, and *Artemia* sp., nauplii, was utilized as food and aeration was readjusted to maintain live food in suspension. Algae concentrate (50 mL, Nanno3600; Reed Mariculture, Pasadena, CA) and yeast (20 g; Lefevre, Milwaukee, WI) were added to 2 ppt saline water (950 mL) and mixed using an electric blender. The resulting algal supplement was then added to each tank each morning (1 mL), providing turbidity as well as nutritious food for the live feed. In addition, algal food supplement was added to tanks throughout the day as well to maintain turbidity between 10 and 20 nephelometric turbidity units (NTU). The system was exposed to 24/7 h/day of light supplied by fluorescent white light bulbs (GE Ecolux Starcoat 32W Office/Prof 3500K) with light intensity at the water surface ranging from ~750–950 Lux, for the duration of the experiment.

Water quality measurements

Water quality parameters were measured in each container daily from 6 to 27 dpf, at which time parameters were measured every other day for the remainder of the experiment (28–47 dpf). Measurements were taken at 9:00 a.m. each morning, and temperature and turbidity measurements were repeated at the end of each day before the final feeding. A multiparameter instrument (YSI, Inc., Yellow Springs, OH) was used to measure temperature (°C), dissolved oxygen (DO, mg/L), pH, and nitrogenous waste (NH₃ and NH₄⁺). A Hach3800 portable turbidimeter was used to measure turbidity (NTU), and a VEE GEE handheld refractometer was used to measure salinity (ppt).

Culture protocol

Zebrafish larvae were stocked to the experimental system at 5 dpf at the density of 100 larvae/L to three replicate containers containing 6 L of 2 ppt saline water. Feeding began at 6 dpf with live rotifers cultured in-house at 20 ppt within a specialized recirculation unit (Aquatic Ecosystems, Pentair). Rotifers were fed to each container four times per day to maintain a density of 140 rotifers/mL. Rotifer density was determined both prefeeding and postfeeding (before addition of rotifers and immediately following addition of rotifers) in the morning and then again in the afternoon, 8–10 h later. Density was determined by collection of a 1 mL

FIG. 1. Rearing system for zebrafish larvae and juveniles until adulthood.

water sample from the water column of each container. The sample was placed in a Petri dish and acidic Lugol's solution (Potassium iodide in 5% acetic acid, 1 mL) was added to the dish to fix the live organisms. The number of individuals was then counted and expressed as per milliliter. Samples were also checked for protozoan growth at this time, and no protozoa were observed.

Containers were siphoned twice per day, before the first and last feedings, to remove fecal material. The volume of water removed was replaced after each siphoning. Water changes (50% water volume was removed and replaced with new water) were performed every 3 days. Density of live food was measured before and after each water change, and containers were supplemented with new live food to reach appropriate density. At 12 dpf, fish from the three containers were counted, sampled, and combined. Individuals were then randomly distributed to six poly-round containers at density 43.3 fish/L. Fish in three containers were then transitioned to live brine shrimp nauplii, while fish in the other three containers remained on rotifers for the duration of the study (46–50 days).

Artemia density was determined following the same methods described earlier for rotifers. Rotifer density in each container was increased to 200 rotifers/mL at 12 dpf as well. *Artemia* density was maintained at 10 nauplii/mL. At 16 dpf, fish density in each container was decreased to 16.7 fish/L. At 22 dpf, fish density in each container was decreased again, to 5.3 fish/L. Rotifer density in each container was then decreased to 100 rotifers/mL, while *Artemia* density remained at 10 *Artemia* nauplii/mL. At 34 dpf, fish began showing signs of sexual maturity and density in each container was decreased to 2.5 fish/L.

Growth, survival, and gonad development

At 6, 10, 12, 16, 20, 27, 34, and 41 dpf, larvae, juveniles, or matured fish ($n=5-12$) from each container were sampled at random for measurement of total length (mm) and weight (mg). Mean weight was natural log-transformed and specific growth rate (SGR) was calculated for both *Artemia*- and rotifer-fed groups at 16, 20, 27, 34, and 41 dpf. The number of fish in each container was recorded at 12, 16, 22, and 41 dpf to determine percent survival. Male and female final weight and length were recorded after fish were spawned (43–46 dpf) ($n=16$). All fish in each tank were sexed at the end of the experiment. *Artemia*- and rotifer-fed groups were sampled for histological analysis of gonad development (two individuals at 16 and 20 dpf and six at 27 dpf). Fish were preserved in formalin, dehydrated, and embedded in paraffin according to standard protocols. Tissue sections were stained with hematoxylin and eosin.

Breeding trials

At 36 dpf, zebrafish in *Artemia*-fed containers and at 39 dpf, zebrafish in rotifer-fed containers appeared sexually mature based on body shape and size. Breeding pairs from rotifer-fed and *Artemia*-fed containers were set up for breeding trials beginning at 36 dpf. Pairs were set in breeding boxes of 2.75 L in zebrafish freshwater recirculation system tanks, where the male and female were separated by a barrier overnight in a normal light/dark (12:12) regime. The following morning, the barrier was removed, and the pair was allowed to spawn naturally for 6 h. Embryos were then col-

lected and transferred to egg baskets in a separate zebrafish recirculation system for incubation (28.5°C). Fecundity, the number of eggs produced in a single clutch, 1 day, from a single female, was measured after breeding pairs were allowed to interact for 6 h. Embryo survival was determined at 24 hours postfertilization (hpf). Twenty breeding pairs were set between 36 and 46 dpf.

Statistical analysis

All percentage data were arcsine transformed before statistical analysis. A pooled *t*-test was used to compare the means of total length, weight, and SGR ($\text{Log}N$ end weight – $\text{Log}N$ initial weight/number of days of growth period $\times 100$) from the *Artemia*-fed and rotifer-fed groups at 16, 20, 27, 34, and 41 dpf. A pooled *t*-test was also used to compare survival percentage between the *Artemia*-fed and rotifer-fed groups at 12, 16, 22, 34, and 41 dpf. A pooled *t*-test was used to compare environmental conditions between the *Artemia*- and rotifer-fed groups over the course of the experiment. A probability of $p < 0.05$ was considered significant and Cohen's *d* (*d*) was used to calculate the standardized difference between means (sample size effect). Cohen's *d* values of 0.5 and greater were considered to have a moderate to large size effect. Significant results of pooled *t*-tests are reported as [*t*-ratio [degrees of freedom] = value, *p*-value = value, Cohen's *d* = value]. All statistical analyses were conducted using JMP 10 software (JMP, Version 10, 1989–2013; SAS Institute, Inc., Cary, NC).

Results

Environmental conditions (Fig. 2)

Artemia-fed groups had significantly higher DO [6.2 vs. 5.2 mg/L, $t(137)=2.28$, $p=0.0239$, $d=0.00281$] and turbidity [21 vs. 16.5 NTU, $t(131)=2.11$, $p=0.0368$, $d=0.01585$] than rotifer-fed groups over the course of the experiment. Both indicators were most likely associated with higher biomass of rotifers than brine shrimp. In one case (12 dpf), oxygen concentration decreased in the morning to 2.8 mg/L in a rotifer treatment container. Fish in the affected tank were concentrated at the surface, but no fish mortality was observed. Rotifer-fed groups had significantly higher pH [$t(137)=3.93$, $p=0.0001$, $d=0.00138$] and NH_3 [$t(137)=8.84$, $p < 0.0001$, $d=0.01630$] than *Artemia*-fed groups over the course of the experiment. There was no significant difference in temperature (°C) [$t(137)=1.58532$, $p=0.1152$, $d=0.00113$] or NH_4^+ [$t(137)=0.48$, $p=0.6302$, $d=0.08239$] between treatments.

Live food density (Fig. 3)

Rotifer and *Artemia* density were both significantly higher in postfeeding samples than prefeeding samples [$t(193)=14.85$, $p < 0.001$, $d=1.45691$ and $t(193)=16.59$, $p < 0.0001$, $d=0.07513$, respectively] throughout the entire experiment. Both prefeeding and postfeeding density of rotifers and *Artemia* were not significantly different between tanks of the same treatment group throughout the course of the experiment. Furthermore, both rotifers and *Artemia* were supplemented during the day, as needed to keep the desired concentrations.

FIG. 2. Water quality for initial exogenous feeding with rotifers (6–12 dpf) and *Artemia*- and rotifer-fed groups (12–41 dpf). (A) Temperature ($^{\circ}\text{C}$), (B) dissolved oxygen (mg/L), (C) turbidity (nephelometric turbidity units), (D) pH. *Artemia*-fed group PR container dissolved oxygen (p -value 0.0239) and turbidity (p -value 0.0368) levels were significantly higher compared with the rotifer-fed PR containers over the course of the experiment. Rotifer-fed group PR container pH (p -value 0.0001) and NH_3 (p -value <0.0001) were significantly higher compared with *Artemia*-fed containers over the course of the experiment. There was no significant difference in temperature or NH_4 . dpf, days postfertilization; PR, poly round.

FIG. 3. Rotifer and *Artemia* density in the PR containers prefeeding and postfeeding throughout the experiment (6–34 dpf). Density of both rotifers and *Artemia* measured after feeding was significantly greater ($p < 0.001$ and $p < 0.001$, respectively), than density measured before feeding.

FIG. 4. Growth data for initial exogenous feeding with rotifers (6–12 dpf) and *Artemia*- and rotifer-fed groups (12–41 dpf). (A) Mean body weight (mg), (B) mean total body length (mm), and (C) mean SGR (% body weight gain/day). Weight and length of *Artemia*-fed groups were significantly higher than rotifer-fed groups at 27 (weight $p=0.03$, length $p=0.0337$), 34 (weight $p<0.0001$, length $p<0.0001$), and 41 (weight $p<0.0001$, length $p<0.0001$) dpf. SGR for *Artemia*-fed groups was significantly higher than rotifer-fed groups at 27 ($p=0.0184$) and 34 ($p=0.003$) dpf. Significant differences denoted by *. SGR, specific growth rate.

Fish growth and survival (Figs. 4 and 5; Table 1)

From 6 to 12 dpf, all fish were fed exclusively rotifers. At 12 dpf, half of the experimental fish of each container were transitioned to *Artemia*, while the rest remained on rotifers. Weight and length of *Artemia*- and rotifer-fed groups were not significantly different from 16 to 25 dpf. However, *Artemia*-fed groups had significantly greater weight and length than the rotifer-fed groups at 27 [weight $t(59)=2.22$, $p=0.03$,

$d=1.68393$; length $t(59)=2.17$, $p=0.0337$, $d=0.32517$], 34 [weight $t(29)=6.12$, $p<0.0001$, $d=10.15054$; length $t(29)=6.23$, $p<0.0001$, $d=1.43262$], and 41 dpf [weight $t(40)=6.96$, $p<0.0001$, $d=16.46293$; length $t(40)=6.44$, $p<0.0001$, $d=1.41035$]. *Artemia*-fed groups reached a mean weight of 446.6 ± 5.8 mg at 41 dpf, nearly 1.6 times the weight of the rotifer-fed groups (281.3 ± 28.4 mg) at 41 dpf. SGR for *Artemia*-fed groups was significantly higher than rotifer-fed groups at 27 [$t(60)=2.43$, $p=0.0184$, $d=0.56818$] and 34 [$t(28)=3.88$, $p=0.0006$, $d=1.17630$] dpf. Female final weight was significantly greater than male final weight [43–46 dpf, $t(14)=2.51$, $p=0.0251$, $d=56.70588$] (Table 1). There was no significant difference [$t(14)=1.15$, $p=0.2702$, $d=0.5675$] in final length between male and female fish at 43–46 dpf. Survival of larvae during initial period (12 dpf) of exclusive rotifer feeding under high fish density (100/L) was $75.6 \pm 10.5\%$. Survival continued to improve in the following intervals and there was no significant difference in percent survival between the *Artemia*- and rotifer-fed groups over the course of the experiment (Fig. 5).

Gonad development (Fig. 6)

The two individuals sampled at 16 dpf weighed 18.0 and 16.8 mg and were 12.5 and 12.7 mm in length. The two individuals sampled at 20 dpf weighed 58 and 38.8 mg and were 17.4 and 16.4 mm in length. All four of these individuals possessed undifferentiated gonads, displaying two types of early germ cells (Fig. 6A, B) according to Maack et al.⁴⁸ Fish sampled from the *Artemia*-fed group at 27 dpf weighed 250.4 and 197.6 mg and were 28.4 and 26.6 mm in length. Both individuals possessed ovaries containing stage I oocytes (Fig. 6C). Rotifer-fed fish were sampled at the same age were 23–27.9 mm in length and 113–217 mg in weight. Histology analysis revealed that individuals from this group also have completed gonad differentiation process and a few previtellogenic oocytes were observed (Fig. 6D). Male gonads of fish fed *Artemia* (from 10 dpf) had germ cells differentiated into primary and secondary spermatocytes at 27 dpf (Fig. 6E). A 1:1 sex ratio was observed in all tanks ($n=6$) across both diet treatments.

Generation time (Table 2)

Breeding pairs were set beginning at 36 dpf, when fish first presented morphological characteristics of sexual maturation. At 43 dpf, an *Artemia*-fed breeding pair were the first experimental fish to successfully produce viable embryos. Embryo survival was determined at 24 hpf (Table 2) and was characterized by high viability (83%–94%). Nine of the twenty breeding pairs set from 36 to 46 dpf produced viable embryos.

TABLE 1. FINAL MEAN BODY WEIGHT AND MEAN TOTAL LENGTH FOR MALE AND FEMALE ZEBRAFISH RECORDED AFTER SPAWNING (43–46 DAYS POSTFERTILIZATION)

	Male	Female
Weight	$361.6 \pm 125.3^*$	$522.7 \pm 146.0^*$
Length	35.3 ± 3.2	36.9 ± 2.4

Female final mean body weight was significantly greater ($p=0.0251$) compared with male zebrafish. There was no significant difference in mean total length between male and female zebrafish. Significant differences denoted by *.

FIG. 5. Average percent survival based on initial stocking density for initial exogenous feeding with rotifers at 12 dpf, followed by average percent survival of rotifer- and *Artemia*-fed groups from each previous survival measurement (12, 16, 22, 34, and 41 dpf). There were no significant differences in percent survival between rotifer- and *Artemia*-fed groups throughout the experiment.

Discussion

Larval/juvenile fish rearing density

For the first time, we used high stocking density (100 larvae/L) in a stagnant saline water system. This initial density at first feeding is much higher than the more moderate

density used by Lawrence *et al.*⁴⁹ (18–25 fish/L) and the low density used by Gomez-Requeni *et al.*⁸ (1.6 fish/L). Under these conditions, we secured zero protozoan growth in the system and eliminated the possible “inside” production of live food. In principal, the common practice is to carry out “fasting,” unfed control treatment in parallel to tested diets

FIG. 6. Early gonad development at three ages, lengths, and weights: (A) 16 dpf, 12.5 mm, 18.0 mg; (B) 20 dpf, 17.4 mm, 58 mg; and (C) 27 dpf, 28.4 mm, 250.4 mg. At 16 and 20 dpf, two types of early germ cells are visible: type 1 (gc1) and type 2 (gc2) according to Maack *et al.*⁴⁸ At 27 dpf, stage I oocytes (I) are visible. Scale bars in all three images represent 50 μ m. (D) Rotifer-fed individual (at day 27 dpf) with a few stage II previtellogenic oocytes present. (E) *Artemia*-fed individual with testis containing primary (PS) and secondary (SS) spermatocytes (25 mm, 159 mg).

TABLE 2. BREEDING PAIRS WERE SET BEGINNING AT 36 DAYS POSTFERTILIZATION, WHEN FISH FIRST PRESENTED GENDER-SPECIFIC MORPHOLOGICAL CHARACTERISTICS OF SEXUAL MATURATION

dpf	Pairs set	Successful pairs	Successful pairs (%)	Eggs produced/female	Mean embryo survival (% at 24 h)	Fecundity
36	3	0	0	0	—	—
41	5	0	0	0	—	—
43	6	1	16.7	41 ± 0	90.2	41 ± 0
44	5	2	40.0	23.5 ± 14.8	94.1 ± 8.3	34
45	5	3	60.0	37.3 ± 43.4	90.0 ± 15.4	52.5 ± 48.8
46	4	3	75.0	22.3 ± 23.4	80.3 ± 34.2	31 ± 25.5

At 43 dpf, an *Artemia*-fed breeding pair were the first experimental fish to successfully produce viable embryos. Embryo survival was determined at 24 hours postfertilization. Average fecundity, the number of eggs produced in one day in one clutch per female up to 6 h after breeding pairs were allowed to interact, is also presented.

dpf, days postfertilization.

to account for possible “growth” of live organisms that might contribute to larval survival at low density.^{50–52} In this study with zebrafish, high density was intended to minimize the contribution of live food “grown in tank.” Several tests with zebrafish larvae kept in similar conditions (algal paste supplement) carried out in parallel to feeding trials resulted in high mortality within 7–9 dpf (data not shown), further evidence that there were no “grown in-tank” organisms or algal supplement feeding contribution.

Low zebrafish density at the beginning of exogenous feeding, with abundant (excessive) supply of food might result in apparent, but negligible, fish growth (4.2 mm length), accompanied by acceptable survival up to 15 dpf.²¹ For instance, Hazlerigg *et al.*⁵³ claimed that when zebrafish at 2.5 fish/L were fed excessively, 100% of fish survived and reached 7.5 mm at 21 dpf, while fish at a density of 15 fish/L suffered 50% mortality. Ribas *et al.*⁵⁴ obtained 35% survival of zebrafish stocked at initial density of 74 larvae/L, whereas 85% survived when stocked at 9 larvae/L. A similar phenomenon can be illustrated based on results of Farias and Certal⁵⁵ where 41% of larval fish survived, but experienced miniscule growth (10 mm after 45 dpf), even when nutritional value of the formulated diet was inadequate.

While fish density (fish/L) was decreased over the duration of the experiment, biomass (mg/L) actually increased, from an initial 24.5 mg/L when density was 100 fish/L to 1116.4 mg/L in *Artemia*-fed tanks when density was 2.5 fish/L at 41 dpf. The biomass of fish/L used in this experiment was significantly higher than what has been observed in literature (Table 3). In fact, at 16 dpf, fish density was 16.7 fish/L with biomass 248.2 mg/L in *Artemia*-fed tanks, more than 65 times higher than the biomass reported by Gomez-Requeni *et al.*⁸ at 16 dpf with 1.6 fish/L (3.8 mg/L).

The method we have adopted, utilizing low salinity, stagnant (static) water with vigorous aeration, and live marine rotifers, is fairly similar to the experimental design by Best *et al.*⁴ and later modified by Lawrence *et al.*⁴⁹ Initial density used by Best *et al.*⁴ was 50 embryos/1.8 L. Fish were stocked into 1.8 L volume containers with 0.2 L of 5 ppt suspension of rotifers with algae (*Nannochloropsis*) addition. The authors measured salinity in the containers and report 5 ppt salinity; however, it is unclear how this salinity was attained in the 1.8 L volume containers and in what volume of water larvae were reared in. Conditions listed in Lawrence *et al.*⁴⁹ differ significantly between text (25 fish/L) and Table 1

(100 fish/L). Authors enlisted 5 ppt salinity for a period of 5–9 dpf. Best *et al.*⁴ named this high salinity period the “polyculture phase” and the 3 days following, the “transition phase.” At 12 dpf (7 days of feeding) in Lawrence *et al.*,⁴⁹ larvae reached 6.3–7.1 mm total body length.

In the study described here, rotifer densities for the majority of the experiment were 10-fold higher than that used by Best *et al.*⁴ (10–16/mL). Moderate rotifer density used was 34–52 rotifers/mL; however, during this period (5–9 dpf), fish density was much lower in this experiment (50 embryos/1.8 L) in comparison to ours (100 fish/L).⁴ *Artemia* density in this study was over three-fold higher (10 nauplii/mL) than the modest densities (three nauplii/mL) commonly used.⁵⁶ This required continuous aeration to maintain suspension of rotifers. Rotifers were continuously provided with algal paste and fish had 24 h access to food.

Gomez-Requeni *et al.*⁸ utilized formulated starter feed (*ad libitum*) for the first 10 days and then supplemented it (at 12 dpf) with *Artemia* nauplii. Even then, fish had only reached 6.8 mm length at 16 dpf when stocked at density as low as 1.6 fish/L. The authors argue that the growth (Fig. 7) achieved can serve as a “reference growth curve for nutritional

TABLE 3. CALCULATED BIOMASS (MEAN ± STANDARD DEVIATION FISH WEIGHT [MG]/L) OF ROTIFER- AND ARTEMIA-FED TANKS IN THESE EXPERIMENTS AT VARIOUS AGES (DAYS POSTFERTILIZATION) THROUGHOUT THE EXPERIMENT

Age (dpf)	Fish biomass (present study; mg/L)		Fish biomass (from literature; mg/L)		
	Rotifer fed	Artemia fed	Ref. ⁸	Ref. ⁹	Ref. ⁴⁶
5	24.5	—	—	—	—
10	69.3 ± 31.3	—	—	0.8	—
12	109.8 ± 12.1	—	—	—	—
16	226.7 ± 28.7	248.2 ± 52.8	3.8	—	—
20	218.8 ± 28.7	218.8 ± 35.6	—	4.0	—
27	708.8 ± 58.4	797.4 ± 39.3	—	—	—
30	—	—	80.0	8.2	770.0
34	516.5 ± 43.3	745.7 ± 21.6	—	—	—
41	703.2 ± 71.0	1,116.3 ± 14.3	224.0	16.6	—

Calculated biomass (fish weight [mg]/L) at different ages from literature sources is also presented.^{8,9,46}

FIG. 7. Mean body weight (mg) of zebrafish from this work (*Artemia* fed) compared to mean total body length of zebrafish achieved in literature through conventional rearing conditions. *Inset:* Zoomed-in area of figure at 10–20 dpf, this work.^{8,9}

experiments.” Other studies that utilized live food exclusively, provided noticeably better growth than studies that utilized commercial diets. However, there are some results achieved with commercial diet utilization, which are exceptional, for instance, larvae at the density of 18 fish/L fed *Artemia* nauplii achieved 14.3 mm, while larvae fed commercial diet exclusively achieved 10.6 mm after 21 days of feeding (26 dpf).⁵⁶ Most of the authors agree that nauplii of *Artemia* are not suitable as the sole first food for zebrafish⁴⁹ and inappropriate food size or quality result in great standard deviation of the mean fish size (Fig. 8).⁶

Depressed growth of zebrafish larvae (4–5 mm body length at 16–23 dpf) jeopardizes meaningful conclusions in many physiological and nutritional experiments.^{57,58} For instance, Guerrero *et al.*⁵⁹ performed studies of digestive enzyme activity in early ontogeny of zebrafish, where fish were exposed to severe malnutrition or simply starvation,

and fish body length did not change from 3 to 22 dpf. At 22 dpf, mean weight of zebrafish larvae was 2 mg, which is negligible compared to 40 mg at 20 dpf in this study. Consequently, the value of the physiological characterization of zebrafish ontogeny is deviating from healthy, growing fish.

Daily growth rate (SGR) of zebrafish for groups fed rotifers and switched to brine shrimp sampled at 12 and 20 dpf were 32% and 40%/day, respectively (Fig. 4). These values are close to that reported for domesticated cyprinids, common and grass carp,⁶⁰ and much higher than in other studies with wild cyprinid larvae (15%–25%/day).^{61,62} Interestingly, maximum relative growth rate was achieved in zebrafish and other cyprinids at the age of 15–25 days, and then declined. Lawrence *et al.*⁴⁹ reported SGR in zebrafish juveniles (80 mg mean weight) at 3%–5.8%/day at 37 days of age before SGR and then steadily declined. In this work, SGR continued at a rate of 5%/day until first maturation (Fig. 4C).

FIG. 8. Mean total body length (mm) of zebrafish from present work (*Artemia* fed) compared to mean body weight of zebrafish achieved in literature through conventional rearing conditions.⁴⁻⁷

Continuous light throughout rearing

For the first time, we combined constant light with live food availability that secured 24-h feeding/growth of fish for the duration of the first zebrafish generation (43–46 dpf). This is the shortest time from fertilization to the viable egg production reported for zebrafish. Early records suggest 90–120 dpf until maturity is reached,⁴⁵ while Aoyama *et al.*⁵ reported a time of 49 dpf for an individual female. The constant light in this study simply doubles the time of feeding as live rotifers (or brine shrimp) are maintained in the water column for the duration of the experiment. At low food density, continuous light has increased several fold survival of larval fish.⁶³ In the direct comparison of the effect of duration of light regime on larvae of another cyprinid fish, tench (*Tinca tinca*), Wolnicki *et al.*²⁴ documented that an increase from 12- to 24-h light with unlimited access to live brine shrimp nauplii after 20 days, resulted in juvenile mean weight of 31.7 and 88.8 mg, respectively. The protocol of continuous light during rearing of larval stages of percids, European perch⁶⁴ and pike perch (*Sander lucioperca*), combined with 2 ppt water,⁶⁵ proved to be successful as well. In other species, advantage of continuous light regime for growth was conferred during early life stages (40% improvement), but juveniles of barramundi (*Lates calcalifer*) appeared to respond to an internal clock and ceased feeding at the time corresponding to darkness.⁶⁶

These results contradict findings by Villamizar *et al.*²¹ who suggested that under constant light from 1 to 30 days, post-hatch zebrafish larvae fed formulated feed, suffered malformations, and had a significantly lower growth rate compared to fish raised under a white light regime (12 h light/12 h dark). However, overall growth rate during the first 12 dpf was negligible (4.5 mm). In this study, fish larvae fed under continuous light grew at the rate of 37% body weight per day and reached 7–8 mm at 12 dpf (Fig. 4). Therefore, we conclude that our results contradict Villamizar *et al.*'s²¹ conclusion that continuous light has negative impacts on rearing zebrafish. The authors found that fish do not survive in the dark, but the rest of the results with the body length between 6 and 7 mm seem to be rather inconclusive. Based on the definition of "sleep" in zebrafish as a minimum of 6 s of immobility, Elbaz *et al.*²³ suggested that fish larvae at 6–8 dpf undergo circadian regulation of 10–30 min sleep per hour. Perhaps in a turbid environment with constant food access, this rhythmic activity is not impaired and continuous light regime becomes beneficial to fish survival and growth.

In some fish species, continuous light during the larval stages negatively impacts growth and metamorphosis,⁶⁷ and in juveniles/adults, continuous light regime is responsible for disruption of gametogenesis.⁶⁸ Photolabile period has been defined by observations of arrested a-vitellogenesis and/or delay of ovulation. In perch (*Perca fluviatilis*), photoperiod is the decisive factor in male gametogenesis, whereas female gametogenesis was overridden by water temperature.⁶⁹ In implementing induced ovulation, we transitioned fish with external signs of maturation to light/dark regime at 36–46 dpf and monitored behavior during the following 1–2 days to record oviposition and fertilization (Table 1).

High density of live food (rotifer/Artemia)

For the first time, we applied high density of rotifers (100–500/mL) and *Artemia* nauplii (10–50/mL) in rearing larval/

juvenile zebrafish, 10 times higher than density commonly used.⁵ Best *et al.*⁴ after initial addition of 340 rotifers/mL at 5 dpf, maintained live food density of 40–50 rotifers/mL. Lawrence *et al.*⁴⁹ state that they used 800/mL of rotifers in the static system of first feeding zebrafish stocked at relatively low density, 18 fish/L, during the period of 5–8 dpf; however, no additional data were provided regarding fish performance.

When experimenting with larval cobia (*Rachycentron canadum*), which are similar in size (3.9 mm) to first-feeding zebrafish, in similar "green water" conditions of live algae and temperature, rotifer density was maintained at 5/mL, and *Artemia* nauplii at 0.2/mL.⁷⁰ However, density of cobia larvae (18 fish/L) was significantly lower than what was used in our study during the first week of rotifer feeding.

Migration and patchiness of zooplankton frequently lead to density in excess of 500 rotifers/L (<1 rotifer/mL) in natural environments.⁷¹ In subtropical eutrophic lakes, rotifer density, such as *Brachionus caudatus*, reached 2000/L, which coincides with low transparency (0.25 m).⁷² Therefore, the density of rotifers used in this study, and previously with bighead and common carp first-feeding larvae in excess of 1000/mL (K. Dabrowski, unpublished data), is within the range encountered in fish habitats and advantageous for growth of particulate feeding larval fish.

Comparison of rotifers and brine shrimp nauplii

For the first time, we tested the performance of larval/juvenile zebrafish fed one of these live diets during the complete life cycle, until maturation. The exception was the initial period of 5 days of exogenous feeding, when all fish were offered rotifers. This comparison encompasses an aspect of physical limitations of prey size (one individual of *Artemia* nauplii delivers energetic value eight times higher than adult rotifer)³⁷ as well as biochemical composition of these organisms (newly hatched brine shrimp nauplii are limited in polyunsaturated fatty acids (PUFA), whereas rotifers are fed with the emulsion specifically enriched in PUFA).

Increased turbidity by algal suspension

For the first time, we exposed larval zebrafish to high turbidity, "green water," a common practice with larvae of marine,^{34,35,73} and freshwater fish^{31,74,75} to increase food organisms' contrast, decrease prey reaction distance, and avoid aggressive interactions, clinging behavior and cannibalism.⁷⁶ Accounts from natural habitats of zebrafish in Asia strongly suggest that this species thrives in conditions of low underwater visibility, frequently just a few centimeters,² and high algal density.⁷⁷ These habitat characteristics allow zebrafish to avoid predators, interspecies antagonistic interactions, and stress without limiting feeding efficiency (visual field of 2–10 mm is sufficient). In temperate natural environments, turbidity (both algal and sediment) reaches 20–100 NTU (Lake Erie).⁷⁸ Although algal turbidity, in particular, was predicted to decrease fish survival and growth in some scenarios,⁷⁸ other studies found a completely opposite effect and contributed this to the significant increase in number of prey consumed.⁷³ The authors associated this higher intake to the relatively transparent *Brachionus* becoming more visible in contrasting algal background in scattered light. Furthermore, higher occurrence,⁷⁹ accelerated growth rate, and higher

survival (recruitment) due to avoidance of predators were described in both marine⁸⁰ and freshwater fish⁸¹ in high turbidity environments. Therefore, fish larvae utilize algal turbidity as a refugium, resulting in more uniform size, as seen in Figure 8.

Kim *et al.*⁸² analyzed conflicting behaviors between feeding and avoidance of the predator and concluded that the latter stimulus produced a stronger and lasting (memorized) response. If predation stress can be substituted for intraspecific antagonistic behavior among larval fish,⁸³ then aggressive interactions will limit food intake and growth. Consequently, increased turbidity and limiting visual field to 0.2–1.2 mm diameter⁸⁴ do not restrict location of the prey, but the distance of <4–5 body lengths apart essentially eliminates aggressive interactions with other conspecifics.⁸⁵ It can also be a function of food size (protozoan of 50 μ m or 3° of visual field)⁸⁶ or prey density (100–400 rotifers/mL; Fig. 3) to minimize search. Transition from larval to juvenile fish size (age) might also result in a “switch” to schooling as the major feeding strategy in high-transparency environments.⁸³

In turbid conditions, it may be argued, zebrafish avoid chronic social stress throughout life,⁸⁷ but the effect of “isolation” in respect to schooling, kin-to-kin and non-kin interactions,⁸⁸ requires further investigation. In effect, the stress level measured as the cortisol concentration response increased 20- to 40-fold in both subordinate and dominant zebrafish in comparison to control.⁸⁷ The effect of stress resulting in growth depression has been associated with increased expression of skeletal muscle myostatin. Certainly, the aspect of diminished stress due to elimination of competition for food and aggressive social interactions in the environment of high turbidity can be quantified in a specifically designed experiment. This positive effect will be magnified in the case of continuous light and access to food.

Physiologically tolerated salinity

For the first time, we have used 1.8–2.1 ppt salinity in the rearing system throughout the entire period of life of the generation. This is lower salinity than what was claimed to result in high performance of zebrafish larvae by Best *et al.*⁴ The latter authors suggested that they reared zebrafish starting at 5 dpf “at water salinities nearly 6 ppt,” although their described methodology contradicts this assertion (200 mL mixture of 5 ppt salinity water containing rotifers was (then) added to a 1.8 L nursery tank” (p. 290). Furthermore, the citation in Best *et al.*⁴ (after Sawant *et al.*⁴⁰) that “zebrafish embryos and early larvae will survive at salinities of up to 14 ppt” is highly inaccurate, as the latter authors clearly stated that in case of zebrafish gastrula-stage embryos exposed to 12 and 14 ppt salinity for 2 h, “the embryos neither gastrulated nor survived.”⁴⁰ Therefore, zebrafish larvae tolerance to salinity and possible stress effects, when measured as an increase in total body cortisol following exposure of zebrafish larva to 2.92 ppt NaCl,^{41,89} needs further investigation. In other species of cyprinid fish, moderate salinity of 5 ppt increased survival (90%–92%) in comparison to control (79%–80%) during embryonic development, and larvae exposed (3 days after hatching) to 2.5–5 ppt survived, whereas those at 7.8 ppt suffered heavy mortality.⁹⁰ El-Gamal and El-Greisy⁹¹ documented the growth rate and survival of common carp raised at 5 ppt during the first 45 days on wild zooplankton

and concluded that survival (91%) was significantly improved in comparison to control fish at 1.5 ppt (62.7%). Weight gain was also significantly improved in increased salinity (2.4 vs. 1.3 g, respectively). Bein and Ribi⁹² demonstrated dramatic improvement in survival of freshwater European perch (*P. fluviatilis*) larvae during 24 days at 0.6–1.2 ppt, in comparison to freshwater. However, 9.8 ppt was lethal for this species. We found salinity 3–3.5 ppt advantageous to rotifers and brine shrimp nauplii viability and “growth promoting” in larval rearing of many freshwater fish, including zebrafish (K. Dabrowski, pers. comm.). Therefore, benefits of rearing freshwater fish larvae at ~2 ppt conditions outweigh possible evidence of stress effect of single, acute exposure. In fact, the method of zebrafish rearing at low salinity described in this study did not have any measurable effect on zebrafish performance over nine generations (M. Miller and K. Dabrowski, unpublished data).

Gonad development

For the first time, zebrafish have produced viable progeny at the age of 43 days; however, this age corresponded to the female size of 0.5 g (Table 1), frequently associated with fish of 65–93 dpf.^{8,46} It has been recognized that reproductive maturation in zebrafish is dependent upon size, not solely age,^{17,93} but the use of age when describing postembryonic zebrafish development has been inappropriately maintained in the scientific literature. Efforts to change this convention across the zebrafish research community⁷ will hopefully extend to studies describing gonad development. Previous work has shown that fish with standard length <10 mm (14–35 dpf) typically have indifferent gonads containing early germ cells, and fish of 10–20 (35–56 dpf) mm typically have juvenile ovaries containing stage I oocytes.^{48,94}

In this study, fish at 16–20 dpf and total length of 12.7 and 17.4 mm retained undifferentiated gonads, despite standard length being longer than 10 mm. Chen and Ge¹⁷ concluded that the definite signs of transition from undifferentiated germ cells to primary-growth oocytes takes place around 28–35 dpf, when female size is 25–50 mg. Regardless of age, sex can be determined in 100 mg (1.8 cm) individuals at 46 dpf using histological analysis.

In this study, an undifferentiated gonad stage was already present at corresponding size at 15–20 dpf, much earlier than described by Chen and Ge.¹⁷ Fish at 27 dpf with total lengths of 26.5 and 28 mm had ovaries containing mostly stage I oocytes. Chen and Ge¹⁷ found that females of this size (26–28 mm), but older (60 and 90 dpf), had ovaries containing large vitellogenic oocytes. This contrast suggests that the rapid growth rate of fish under our conditions causes gonad development to progress quicker in time (as we observed reproductive maturity earlier than previously recorded), yet at larger sizes than would be expected under a slower growth rate. This phenomenon of rapidly growing fish reaching distinct stages of gonad development at younger ages and larger sizes was also reported for the closely related common carp *Cyprinus carpio*.⁹⁵ It can be concluded that when describing gonad development, both age and size must be considered. In effect, as has been pointed out in respect to many fish species, providing solely age (dpf) leads to highly inaccurate conclusions regarding overall morphological transformation (metamorphosis) of teleost fishes.

Maturation, fertility, and generation time

First spawners (43–46 dpf) produced numbers of eggs that were not notably different from previous data in the literature.^{10,96} Von Hertel *et al.*¹⁰ reported within and between families, variation in daily egg production from 40 to 89 and hatching rate from 19% to 65%. The “large” size category of females in their study produced 38.7 eggs per daily clutch and that female size corresponds to the size of females in this study (36.9 mm) (Table 2).¹⁰ However, the results of Uusi-Heikkilä *et al.*⁹⁶ were potentially biased by the fact that the “large” females might have spawned several times before the age of 180 dpf when the comparison to “small” (virgin) females was carried out (~18 eggs/clutch/day). Lawrence *et al.*⁴⁹ concluded that the proportion of zebrafish females of 57 dpf (0.22–0.25 g mean weight) that successfully spawned was <18% and the mean number of embryos per clutch was less than 45 ± 10 (50% viable). This again points out to the dependence of zebrafish puberty and fecundity primarily on the size (growth) rather than age.¹⁷ In the longest described studies of zebrafish controlled breeding, Shinya and Sakai⁴⁷ established that over the 13 generations of inbred zebrafish (polymorphism dropped from 62% to 5%), neither the mean number of egg per clutch nor fertilization rate (over 90% at 3 dpf) changed significantly. Therefore, we conclude that the quality of females produced following this new rearing protocol did not impact their reproductive efficiency.

General Conclusions

The combination of novel rearing conditions utilized in this work resulted in experimental zebrafish achieving greater growth and a shorter generation time than what has previously been described in the literature, while experiencing no significant decrease in survival. We have explained the differences between our proposed modified protocol and the very variable conditions utilized in previous publications, and have suggested that the four key modifications used in this study, light, salinity, turbidity, and food availability, when used in concert, led to superior growth, survival, and within-population (cohort) uniformity in size. These metrics do not suggest negative impacts on fish performance compared to this approach. However, we recognize that some proposed modifications, such as constant light, cannot be used in some studies, such as those addressing behavioral rhythmicity. Further research may examine the effects of each proposed modification separately and provide quantitative measures of their independent impacts on zebrafish performance. Based on the presented results, eight generations of zebrafish can be produced in 1 year following this methodology, reducing the time required for multigenerational selection, a trait useful in various research fields.

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Disclosure Statement

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