Effects of Diet and Social Housing on Reproductive Success in Adult Zebrafish, *Danio rerio*

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Abstract

Zebrafish (*Danio rerio*) have proven their efficiency as an animal model for genetics and development, but their nutrition and housing requirements continue to elude researchers. Diet and housing density were predicted to affect weight change and reproductive success in 120 days postfertilization (dpf) zebrafish, and growth performance of their progeny. Fish were fed one of four diets, each utilizing a different primary protein source (fish meal [Zeigler™], algae, or insect), while being housed 3.3 or 6.6 fish/L for 3 weeks. Clutch size, viability, and larval development of their progeny were monitored out to 10 dpf. All diets were sent out for proximate nutrient analysis and fatty acid profiles to understand how diet compositions affect reproduction. We found that diet and housing proximity affected adult fish weight and larvae growth; diets composed of higher levels of protein and polyunsaturated fatty acids (specifically arachidonic acid [AA] and eicosapentanoic acid) allowed fish to gain weight and produce healthy larvae. Fish housed at higher densities produced smaller embryos, but larger larvae than those housed at lower densities. These findings imply that significant effects of a modified stimulus are exhibited after relatively short periods.

Keywords: nutrition, social housing, fishmeal, algae, insect

Introduction

THE ZEBRAFISH (*DANIO rerio*) has become a staple animal model in biomedical research for many reasons, including their small size and ease of husbandry.^{1–3} Compared to the husbandry needs of other traditional animal models, zebrafish life-support systems promote higher efficiency usage by housing larger amounts of animals in smaller spaces; allowing researchers to customize social housing and diet based on breeding needs. Robust, predictable breeding and acquisition of viable embryos are among the most important management goals in any zebrafish facility.^{2,3} Growth performance of larval-stage fish is vitally important for most sectors of zebrafish research,⁴ but is highly dependent on various biotic and abiotic variables (Table 1). Therefore, improving the fish's housing conditions and diet should increase the fish's research utility. Diet^{5–7} and housing density^{8,9} have been shown to affect reproductive fitness and behavior, but the goal of the current study is to explore how varying housing conditions and diet regimes affect reproduction and subsequent larval development in captive fish.

The zebrafish is widely distributed across freshwater habitats in southern Asia.^{2,10,11} In the wild, they display a

range of social behaviors, but typically tend to associate in groups that vary in size and activity in relation to habitat type.¹² In captivity, social structuring and territoriality are common and density-dependent. These interactions take place within and between sexes, and are typically mediated via aggression. Both sexes exhibit mate choice when spawning,^{2,13,14} and can recognize their own kin to promote higher genetic diversity when breeding.¹⁵

Reproduction in the wild is relatively complex and depends on light cycles and circadian rhythms.^{15–17} Relatively little is known about natural spawning behavior in wild habitats. However, reproduction in research facilities has been reasonably well-characterized.^{8,14,16,18–20} Successful spawning is heavily dependent on factors like diet and social behavior,^{2,18} so these variables can be manipulated to improve reproductive efficiency. For example, breeding success (specifically spawning rate and viability) could be maximized by housing fish at higher densities (12 fish/L) to reduce hierarchical effects. Housing fish at lower densities (6 fish/L) could therefore intensify their social hierarchy via increased cortisol levels.^{21,22} This increases dominance of one or two individuals, which increases male-male competition and reduces female mate choice, and subsequently, clutch size

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TABLE 1. LIST OF ABIOTIC AND BIOTIC FACTORS THAT AFFECT REPRODUCTIVE SUCCESS IN ZEBRAFISH

		<i>High success</i>
Abiotic	Light cycles	Initiate spawning as lights approach full intensity (mimic rising sun)
	Timing	Initiate spawning at start of daylight
	Housing—group vs. single	House fish with conspecifics to reduce stress and egg burden in females
	Depth of water	Mimic beach incline
	Enrichment	Include plant for subordinate fish to hide
	Water quality	Temp: 28.5°C; pH: 7–8; NO ₂ : 0 ppm; NO ₃ : 0–5 ppm; NH ₄ : 0–10 ppm; Hardness: 100 mg/L; Salinity: 0.25 ppt
Biotic	Competition for mates	Males compete for females with larger bellies
	Frequency of breeding	Breed each fish once/week
	Sex hormones	Provide slits in breeding tanks for water circulation
	Ratio of male to female	1m:2f
	Nutrition	High protein content, high fat/lipid content, high digestibility (ideal ranges remain unknown)
	Feeding frequency	To satiation or 5% body weight, up to three times daily
	Genetic manipulation	dependent on degree

Third column indicates parameters that yield high success.

consistency.¹⁴ Experimenting with varying housing densities (lightly housed vs. densely housed) could help facility managers to understand the ecological mechanisms at play in zebrafish social behavior.

Zebrafish are opportunistic, omnivorous feeders, consuming zooplankton, insects, and phytoplankton,¹⁶ but their precise nutritional requirements are not known.^{2,3,23} However, the fish display improved growth and survival performance when their diet includes high levels of protein and fats, including polyunsaturated fatty acids (PUFAs).^{24,25} Diet composition has differing effects on breeding efficiency.^{5,17,26,27} Facility managers have been increasingly favoring processed diets with high protein concentrations, such as those developed for similar species to zebrafish (e.g., sea bream).^{17,23,28–30} While good nutrition is of obvious importance for the husbandry of any captive organism, few studies have been done to determine the specific nutritional requirements of zebrafish.^{5,31} Diets composed of primarily fish protein³⁰ have been most commonly evaluated in zebrafish, but the effects of other protein sources (such as those that occur in their natural diets) have only recently been studied.^{6,32} The effects of fatty acids on zebrafish reproduction have been partly understood,^{7,24,33} but the impacts of variable fatty acid concentrations in zebrafish diets has not been adequately considered.^{7,25,33} It is known for marine fish that eicosapentanoic acid (EPA), arachidonic acid (ARA), and docosahexaenoic acid (DHA) are necessary for both males and females to produce healthy gametes, which require high lipid content. Healthy females use these lipids mostly for their oocyte production, which necessitates the lipids high demand.³⁴

Since large clutch size is coveted by biomedical researchers, breeding females can produce higher numbers of eggs more frequently if they are consuming diets that have high concentrations of fatty acids, specifically n-3 and n-6 PUFAs. Several studies have shown that 1%–1.5% of sparid and salmonid diets should be composed of PUFAs, specifically EPA, ARA, and DHA.^{7,35} Cyprinids (including zebrafish), salmonids, and sparids reproduce similarly, which infers that they require the same amount of lipids in their diets. Therefore, further studies must be done to

understand the impacts of fatty acids on zebrafish reproduction, specifically.

Ultimately, improved nutrition and optimized housing density can result in higher egg quality, as measured by clutch size, egg diameter, and yolk sac size.^{14,36,37} For example, larger fish will tend to produce larger eggs, but size does not necessarily correlate positively to fish health.³⁷

The goals of this study were to measure the effects of: (1) each of three diets with differing protein sources (fishmeal, algae, and insect) and PUFA concentrations, and (2) two housing densities (lightly group-housed [LGH] or densely group-housed [DGH]) on weight change, larval growth and development, and viability. Of the three diet treatments, we predicted that a fishmeal-based diet would result in the best performance and healthiest offspring because it has a higher protein and PUFA content than the algae or insect diets. Also, we predicted that fish that were housed more densely would exhibit higher reproductive fitness after spawning, and would subsequently produce healthier offspring due to the increased level of contact with conspecifics, thereby reducing social hierarchies.

Materials and Methods

Acclimation: baseline for experiment

One hundred sixty-two captive-raised (12.68 fish/L density) adult zebrafish (Casper EKK, 120 days postfertilization [dpf]) were randomly selected and sex-segregated (81 males, 81 females). All fish were placed into a self-contained iSpawn™ apparatus³⁸ overnight to promote male courtship behavior and female ovulatory behavior in preparation for group spawning. The next morning, the iSpawn divider was removed and fish were allowed to breed in shallow water for 2 h to clear old oocytes from female fish, and also to ensure that breeding was achieved. A feed ration of 5% body weight³² was developed from the average weight per fish (0.3 g) for each housing treatment. Fish were then randomly placed in eighteen 1.8 L tanks in aggregations of 6 (3m:3f = lightly housed) or 12 (6m:6f = densely housed), and placed on recirculating Aquaneering™ rack systems. It is known that zebrafish are normally housed at 12.68 fish/L in many

facilities, but for the purposes of this study, a density of 6.6 fish/L was considered densely housed instead of 12.68 fish/L. A complete description of the husbandry and environmental conditions in housing for the fish used in these experiments is available as a collection in protocols.io (dx.doi.org/10.17504/protocols.io.mrjc54n). All fish were fed Gemma Micro 300™ twice daily for 7 days. This acclimation period was implemented to transition fish to their new experimental housing density. It also acted as a baseline to reduce stress before altering the fish's diet for 3 weeks. After the acclimation period, fish were bred to clear out existing oocytes (that may have developed from Gemma) and then transitioned to their experimental diets.

Feeding trials: experimental diets

After the acclimation period, tanks were assigned one of three experimental dietary treatments: (1) Zeigler™ fish-meal based pellet, (2) New Life Spectrum™ Algaemax wafers, or (3) Enviroflight™ insect-based meal, and one of the two housing treatments: (1) LGH, or DGH. Average weights of fish/tank were used to calculate food ration (5% body weight), administered via a hand-held fish feeder (Danio-Lab™) which was calibrated to feed the appropriate amount of food (Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/zeb). Fish were fed their assigned experimental diet twice daily (2.5% in the morning, 2.5% in the afternoon) for 3 weeks. Feed ration was re-calculated each week depending on fish weight, which was then used to re-calibrate the feeder; thus ensuring that fish were fed 5% of their body weight even if they were growing.

After 3 weeks, fish were weighed and set up in crossing tanks 12 h before breeding, bred for 2 h, and then weighed again to track weight loss and gain due to breeding. Two-tailed paired *t*-tests were computed to determine if there was a significant difference between initial weight and final weight after 3 weeks (including breeding events) and for the 2 week interval between breeding events for all diets. Embryos were collected and counted to track viability in the same manner as the control treatment over the next 24 h. Embryos were housed in petri dishes (100 mm diameter) at 28.5°C for survival and growth to 10 dpf. Live rotifers were administered to larvae at 5 dpf to encourage natural foraging behavior and were replenished every day or *ad libitum*. Images of each clutch were taken at 1, 5, and 10 dpf to compare growth rates and development. ImageJ was used to quantify embryo diameters and larval lengths using scale bars on each picture. Tricaine methanesulfate (MS-222) was used (0.168 mg/mL) to anesthetize larvae at 5 and 10 dpf to facilitate imaging.^{3,39}

Clutch size and viability

Student's *t*-tests and two-way analyses of variance (ANOVAs) were computed (to determine housing and diet differences, respectively), for clutch size and viability.

Fish weight change

Two-tailed paired *t*-tests were computed to determine if there was a significant difference between initial weight and final weight.

Embryo size

Student's *t*-tests and two-way ANOVAs were computed (to determine housing and diet differences, respectively), for yolk sac size and chorion diameter.

Larvae length

Student's *t*-tests and two-way ANOVAs were computed (to determine housing and diet differences, respectively), for length at 5 dpf and length at 10 dpf.

Nutritional analysis

Two hundred grams of each diet was sent to Litchfield Analytical Services, Litchfield, MI to gain proximate nutrient analysis (Table 2) and fatty acid profiles (Table 3). Ingredient compositions of each diet are detailed in Supplementary Table S2.

Results

After running a Shapiro-Wilk test, all data was normally distributed, so parametric tests were done. All statistics were run using GraphPad Prism.

Clutch size and viability

There was no significant relationship of housing density and diet on clutch size ($F(18)=0.8372$, $p=0.571$). Neither housing density nor diet treatment had an effect on viability of embryos after 24 h ($F(3,18)=1.058$, $p=0.3915$).

Fish weight change

There was a significant diet effect on average weight change between breeding events 2 and 3 in light housing for only Zeigler ($t_{\text{Zeigler}}(2)=48.44$, $p=0.0004$; Fig. 1a), but not for algae or insect ($t_{\text{algae}}(2)=2.503$, $p=0.1294$; $t_{\text{insect}}(2)=2.952$, $p=0.0982$; Fig. 1b, c). There was a significant diet effect on average weight change between breeding events 2 and 3 in dense housing for Zeigler and algae ($t_{\text{Zeigler}}(2)=5.636$, $p=0.0301$; $t_{\text{algae}}(2)=11.83$, $p=0.0071$; Fig. 1f, g), but not for insect ($t_{\text{insect}}(2)=0.8046$, $p=0.5055$; Fig. 1h). There were no significant differences between average initial weight and average final weight over the entire 3-week feeding trial (including breeding events) for light housing ($t_{\text{Zeigler}}(2)=1.514$, $p=0.2692$; $t_{\text{algae}}(2)=0.08$, $p=0.9435$; $t_{\text{insect}}(2)=3.733$, $p=0.0649$; Supplementary Fig. S1a–c). For dense housing, there was a significant weight gain in fish that were fed only Zeigler ($t_{\text{Zeigler}}(2)=4.645$, $p=0.0434$; Supplementary Fig. S1d), but no differences in algae or insect ($t_{\text{algae}}(2)=3.876$, $p=0.0606$; $t_{\text{insect}}(2)=0.2046$, $p=0.8568$; Supplementary Fig. S1e, f).

Embryo size

Fish that were housed densely produced embryos with significantly larger yolk sac diameters than fish that were housed lightly for only the insect diet ($t_{\text{insect}}(235)=7.685$, $p<0.0001$, Fig. 2a). Chorion diameter was significantly higher in progeny of fish that were densely housed than those housed lightly for all diets except algae ($t_{\text{Zeigler}}(339)=4.269$, $p<0.0001$; $t_{\text{insect}}(284)=4.208$, $p<0.0001$; Fig. 2b).

TABLE 2. PROXIMATE NUTRIENT ANALYSIS FOR EACH DIET

<i>Diet</i>	<i>Gemma™</i>	<i>Zeigler™</i>	<i>New Life Spectrum™</i>	<i>Enviroflight™</i>
<i>Type of food</i>	<i>Pellet</i>	<i>Pellet</i>	<i>Wafer</i>	<i>Meal</i>
<i>Type of protein</i>	<i>Fish-meal</i>	<i>Fish-meal</i>	<i>Algae</i>	<i>Insect</i>
Moisture (%)	8	8.39	7.02	2.66
Dry matter (%)	92	91.61	92.98	97.34
Crude protein (%)	59	54.92	36.98	52.19
Crude fiber (%)	0.2	0.71	4.18	7.34
Crude carbohydrates (%)	5.9	11.10		10.55
Fat (%)	14	14.95	11.51	15.40
PUFA (%)	5.18	2.691	1.3812	3.08
TDN (%)		79.98		77.32
Phosphorus (P) (%)	2	1.30	0.61	1.03
Calcium (Ca) (%)	1.5	1.89	2.83	3.08
Potassium (K) (%)	0.3321	0.59	0.73	1.32
Magnesium (Mg) (%)		0.17	0.43	0.32
Sodium (Na) (%)	0.7	0.78	1.41	0.16
Copper (Cu) (ppm)	10	51	25	11
Iron (Fe) (ppm)	220	586	1200	183
Zinc (Zn) (ppm)	170	126	67	126
Manganese (Mn) (ppm)		65	57	104
Ash	13		16.27	
Sulfur (%)			0.8	

PUFA, polyunsaturated fatty acids; TDN, total digestible nutrients; ppm, parts per million.

TABLE 3. FATTY ACID PROFILES FOR EACH DIET, DETAILING CHEMICAL PROPERTIES AND PROPORTIONS (%) OF OMEGA-3 (N-3) AND OMEGA-6 (N-3) FATTY ACIDS

					<i>Gemma</i>	<i>Zeigler</i>	<i>New Life Spectrum</i>	<i>Enviroflight</i>
					<i>Pellet</i>	<i>Pellet</i>	<i>Wafer</i>	<i>Meal</i>
	Omega?	Carbons	Double Bonds	Saturation	Fish-meal	Fish-meal	Algae	Insect
Alpha linoleic acid	n-3	18	3	Polyunsaturated				0.75
Arachidonic acid	n-6	20	4	Polyunsaturated	0.85	0.48	1.03	0.00
Capric acid		10	0	Saturated				0.70
Eicosadienoic acid, eicosatrienoic acid	n-6	20, 20	2, 3	Polyunsaturated	0.18	0.34	0.30	
Eicosapentaenoic acid	n-3	20	5	Polyunsaturated	3.29	5.90	0.73	0.87
Eicosenoic acid		20	1	Monounsaturated				0.38
Erucic acid		22	1	Monounsaturated	6.12	4.14	10.11	
Gadoleic acid		20	1	Monounsaturated	2.39	3.70	1.89	
Gamma linoleic acid	n-6	18	3	Polyunsaturated				0.25
Lauric Acid		12	0	Saturated	1.46	6.32	10.07	34.10
Lignoceric acid		24	0	Saturated	1.39	1.31	1.84	0.00
Linoleic acid	n-6	18	2	Polyunsaturated	29.83	10.26	8.09	18.05
Linolenic acid	n-3	18	3	Polyunsaturated	3.13	1.44	2.13	
Myristic acid		14	0	Saturated				7.58
Nervonic acid		24	1	Monounsaturated	15.64	3.87	6.93	
Nonadecanoic acid, arachidic acid		19, 20	0, 0	Saturated	0.85	1.45	2.36	
Oleic acid		18	1	Monounsaturated	12.67	23.15	14.87	15.30
Palmitic acid		16	1	Monounsaturated	16.57	23.43	24.92	3.00
Palmitoleic acid		16	1	Monounsaturated	1.77	8.67	10.66	
Stearic acid		18	0	Saturated	3.58	5.39	3.88	2.64
Unknown								2.50
Total n-3 PUFA					6.42	7.34	2.86	1.62
Total n-6 PUFA					30.86	11.08	9.42	18.30
n-3/n-6					0.21	0.66	0.30	0.09
% PUFA					37.28	18.42	12.28	19.92
Decimal					0.37	0.18	0.12	0.20
Total% PUFA					62.44	81.43	87.53	63.70

PUFA, polyunsaturated fatty acids.

FIG. 1. F1 weight change between breeding events for each feeding and housing treatment. Each *colored line* (dotted or solid) represents one tank (LGH: 6 fish each; DGH: 12 fish each) from each treatment. *Horizontal solid orange lines* indicate the initial average weight of fish per tank. *Vertical solid black lines* indicate growth intervals (d129: start of foreign diet treatments; d136–137: second breeding event, end of week 2; d138–143: end of week 3; d150–151: third breeding event, end of week 4). *Arrowheads* indicate final weight (averaged from each tank). *Asterisks* indicate statistical significance. (a–c) LGH fish fed Zeigler, algae, and insect, respectively. *Dotted black line* indicates the average weight change of fish per tank. (d–f) DGH fish fed Zeigler, algae, and insect, respectively. *Solid black line* indicates the average weight of fish per tank. DGH, densely group-housed; LGH, lightly group-housed. Color images available online at www.liebertpub.com/zeb

In addition, differences between diets within housing treatments showed several *post-hoc* relationships. For fish housed lightly (LGH), Zeigler and algae were significantly larger than insect yolk sacs ($q_{z,i}(540)=6.787$, $p<0.0001$; $q_{a,i}(540)=9.618$, $p<0.0001$; Supplementary Fig. S2a). Zeigler chorions were significantly smaller than those of algae ($q_{z,a}(951)=4.395$, $p=0.0055$, Supplementary Fig. S2b). For fish housed densely (DGH), no significant relationships were observed in yolk sac diameter (Supplementary Fig. S2a). Insect chorions were significantly larger than those of Zeigler and algae ($q_{i,z}(951)=5.342$, $p=0.0005$; $q_{i,a}(951)=6.883$, $p<0.0001$, Supplementary Fig. S2b).

Larval length

Housing differences did not affect total body length of 5 dpf larvae for any of the diets ($t(493)=0.6572$, $p=0.5114$, Fig. 3a). Total body length at 10 dpf was significantly higher in progeny of fish that were lightly housed than those housed densely for only insect ($t_{insect}(113)=3.586$, $p=0.0005$; Fig. 3b).

In addition, differences between diets within housing treatments showed several *post-hoc* relationships. For fish housed lightly (LGH), Zeigler and algae larvae at 5 dpf were significantly larger than insect larvae ($q_{z,i}(414)=6.656$, $p<0.0001$; $q_{a,i}(414)=4.255$, $p=0.0078$, Supplementary

FIG. 2. Main effects of diet treatments (*blue*: Zeigler; *green*: algae; *tan*: insect) and housing treatments (LGH; DGH) on F2 embryo size. *Brackets* signify significance after paired two-tailed *t*-tests. **(a)** Yolk sac diameter at 1 dpf; $n=100 \pm 10$ for each data set. **(b)** Chorion diameter at 1 dpf; $n=100 \pm 10$ for each data set. dpf, days postfertilization. Color images available online at www.liebertpub.com/zeb

Fig. S3a). Insect larvae at 10 dpf were significantly larger than only Zeigler ($q_{i,z}(394)=3.795$, $p=0.0207$, Supplementary Fig. S3b). For fish housed densely (DGH), Zeigler larvae at 5 dpf were significantly longer than algae and insect ($q_{z,a}(414)=5.141$, $p=0.0009$; $q_{z,i}(414)=7.584$, $p<0.0001$, Supplementary Fig. S3a). Diet didn't cause any significant *post-hoc* differences in 10 dpf larvae (Supplementary Fig. S3b).

Nutritional analysis

Protein was highest in the control diet composed of fish-meal (Gemma), but fat and fiber were highest in the diet composed of insect meal. Gemma had the lowest carbohydrate composition. The algae diet had the highest iron (Fe) composition and the smallest fat composition. Other trace minerals such as phosphorus (P), calcium (Ca), potassium (K), magnesium (M), and sodium (Na) were also analyzed, and no significant trends were seen between diet treatments. Fatty acid profiles of each diet (except Zeigler) revealed a low n-3/n-6 ratio, and all diets were composed of a diverse mix of saturated, monounsaturated, and PUFAs (Table 3).

Discussion

We found that both diet and housing density had effects on the average weight of F1 fish and growth in the F2 generation,

but not on viability or clutch size. It is interesting to note that only two-thirds of all LGH fish actually spawned during the last breeding event, but DGH fish from each diet (except algae) spawned completely, which could support the theory of reduced hierarchy in denser housing.¹⁴ When considering housing density alone, all tanks gained weight over the 2-week period between breeding events, but this was not statistically significant for LGH algae, LGH insect, and DGH insect (Fig. 1). Furthermore, fish that were housed lightly shared a common trend of gaining more weight in the third week than the second (Fig. 1a–c; Supplementary Fig. S1a–c). This suggests that after fish bred (specifically females), they replaced the weight they lost when spawning in the first week, and then were able to build up their weight stores more so in the subsequent weeks. All three diets showed this trend, which indicates that housing fish in groups of six enabled them to consistently gain weight in this pattern, and did indeed have an effect on weight fluctuations. Only the fish fed Zeigler gained enough weight to surpass their initial weight after 3 weeks of time (Supplementary Fig. S1a, d). Fish fed insect and algae did gain weight between breeding events, but did not retain any additional weight than their initial weight.

Surprisingly, fish housed densely did not show this trend, except in fish fed Zeigler. Fish did gain weight between breeding events and ultimately surpassed their initial weight

FIG. 3. Main effects of diet treatments (*blue*: Zeigler; *green*: algae; *tan*: insect) and housing treatments (LGH; DGH) on F2 larvae size. Brackets signify significance after paired two-tailed *t*-tests. **(a)** Total length of larvae at 5 dpf; $n = 50 \pm 10$ for each data set. **(b)** Total length of larvae at 10 dpf; $n = 50 \pm 10$ for each data set. Color images available online at www.liebertpub.com/zeb

at the end of the feeding trial. Fish that were fed algae gained significant weight in the interval between breeding events, but eventually weighed less than their initial weight before breeding. This confirms that the algae diet did not have enough nutrients for an omnivorous cyprinid to promote weight gain over time, especially in broodstock.⁴⁰ The insect diet had a similar effect, in which fish only slightly gained weight between breeding events, and maintained their initial weight at the end of the experiment. Fish housed densely had little consistency in trend between d136 and d150, but on d152, all tanks re-gained consistency with a clear loss or gain in weight. When referring to the entire feeding trial (including breeding effects), diet performance did not differ across housing density. Fish fed Zeigler gained weight, fish fed algae lost/maintained weight, and fish fed insect lost weight. These findings confirm our hypotheses.

Nutritional analysis of each diet supported these trends, as protein and fat only accounted for 36.98% and 11.51%, respectively, of the algae diet's composition (Table 2). Very little is known about the nutrient requirements of zebrafish, but based on studies in similar families of bony fishes they seem to require a diet with at least 45%–55% protein and 1%–1.5% PUFA to promote high breeding success.^{32,35,40} The range accounts for the striking difference between males and females. Fatty acids are a constituent of the fat component, and all of the diets were well within the PUFA guideline, except for the algae diet (Table 3). Additionally, all four of

the diets had n-3/n-6 ratios of below 0.8, which has been proven to result in high breeding success.^{24,25,41} It is unknown how all of the lipids within each diet are interacting within the fish, especially in the female's ovaries, but it is reassuring that the standardized diet used in many labs presently has levels of PUFA exceeding the guideline of 1.5%. Moreover, fish could even be pushed to breed several times per week (as opposed to once a week or twice a month). This would create a high level of stress, which could also prevent the fish from breeding well. Stress is not well understood in zebrafish, but this could cause a significant reduction in breeding efficiency, egg viability, and clutch size. Conversely, the percentage of PUFA in the algae diet (1.3812%) was the lowest of the four tested (Table 2), which could explain their poor breeding performance. It is composed almost exclusively of algae derivatives (Supplementary Table S2), and is therefore not suitable for omnivorous fish, particularly those housed in labs and managed as broodstock.

These data confirm that completely changing zebrafish broodstock diets have significant effects, even over small periods of time (e.g., 1 week). The fish used were only 4 months old, and were not yet adults, so normal growth could have still been happening. Extending the feeding trials out to several months as opposed to 3 weeks could show an initial high growth rate until the fish reach a "plateau" in which they are no longer growing. This means that fish would be healthier due to the rapid turnover of most foodstuffs (input)

to gametes (output). Energy retained would go into homeostatic processes, like digestion, respiration, and immunity. Similarly, if fish were fed each diet from larvae to adult, further trends in the life-stage transition phases (larvae to juvenile; juvenile to adult) could explain whether each diet is better for the fish for the duration of their life or if a specific diet favors one stage over another. For example, comprehensive diets rich in protein and PUFAs (like Gemma and insect) fed throughout the fish's life should result in leaner, healthier adults that produce embryos with high survival, viability, and clutch size.

Housing density had effects on embryo size and larval development for each of the diets. Furthermore, relationships between each diet and housing type were observed (Supplementary Figs. S2 and S3), but will not be discussed here. The abrupt LGH larvae growth between 1 and 10 dpf was only seen for the insect diet. DGH fish produced larger embryos and larvae than LGH fish for the other three diets (Figs. 2 and 3). This striking difference suggests that both housing densities produce favored progeny size, depending on the desired experimental use by researchers. Specifically for the insect diet, housing fish in dense aggregations will produce large embryos and small larvae, which facilitates CRISPR injections or assays that need to be done with embryos. Conversely, housing fish at lower densities will produce small embryos and large larvae. This is an advantage for survival assays or drug treatments in which larval development is the main focus.

Zebrafish are very opportunistic feeders, but the ones used in this study were raised in a captive environment, and being subjected to an unusual food source could have induced a stress response. Relatively little is known about how stress affects breeding, but nutritional deficiency and abrupt changes can instigate a stressful situation, which can then drastically affect reproductive success and health.^{42–44} Further research is needed to understand how nutrition and social proximity interact with cortisol to ensure zebrafish in laboratories remain healthy and consistently produce embryos of the best quality.

Limitations of the Study

There were two major limitations to this study. First, there were twice as many fish housed densely than there were lightly. This could have caused the study to be underpowered even though all Shapiro-Wilk tests passed normality. Inconsistencies in breeding events could have caused high variability between tanks, but our results seem to confirm our hypotheses. Second, this study did not adequately run any tanks on the Gemma diet in parallel to the experimental diets. The week-long acclimation period was designed to act as a baseline which would reduce stress and allow all fish to reach a similar starting point before being switched to one of the experimental diets. Running Gemma tanks as a true control would have made the results much stronger, but the authors argue that drastic changes in the fish's weights were seen within the relatively short period of time that they were shifted off of their normal diet, which coincides with the authors' hypotheses.

Conclusions

This study conclusively showed that short term effects of fish weight and larval development can be seen early after

modifying zebrafish husbandry conditions. Nutrient requirements for zebrafish housed in laboratory conditions are still mostly unknown. Husbandry conditions are often carefully controlled, with a focus on ultimate breeding success. This promotes high breeding efficiency and reproductive success, but developing a diet with ideal levels of necessary components will likely further improve zebrafish breeding potential.

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Disclosure Statement

No competing financial interests exist.

References

- Sciarra J, Tyler A, Kolb A. A gelatin-based diet for oral dosing juvenile to adult zebrafish. *Lab Anim Sci Prof* 2014; 6:32–35.
- Lawrence C. The husbandry of zebrafish (*Danio rerio*): a review. *Aquaculture* 2007;1:1–20.
- Westerfield M. The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish, *Danio rerio*. University of Oregon Press, Eugene, OR, 2000.
- Zon L, Peterson R. In-vivo discovery in the zebrafish. *Nat Rev Drug Discov* 2004;1:35–44.
- Markovich M, Rizzuto N, Brown P. Diet affects spawning in zebrafish. *Zebrafish* 2007;1:69–74.
- Siccardi AJ III, A, Garriss H, Jones W, Moseley D, D'Abramo L, Watts S. Growth and survival of zebrafish (*Danio rerio*) fed commercial and laboratory diets. *Zebrafish* 2009;3:275–280.
- Jaya-Ram A, Kuah M, Lim P, Kolkovski S, Shu-Chien A. Influence of dietary HUFA levels on reproductive performance, tissue fatty acid profile and desaturase and elongase mRNAs expressing in female zebrafish *Danio rerio*. *Aquaculture* 2008;3–4:275–281.
- Castranova C, Lawton A, Lawrence C, Baumann D, Best J, Coscolla J, *et al.* The effect of stocking densities on reproductive performance in laboratory zebrafish, *Danio rerio*. *Zebrafish* 2011;3:141–146.
- Carfagnini A, Rodd H, Jeffers K, Bruce A. The effects of habitat complexity on aggression and fecundity in zebrafish (*Danio rerio*). *Environ Biol Fishes* 2009;3:403–409.
- Engeszer R, Patterson L, Rao A, Parichy D. Zebrafish in the wild: a review of natural history and new notes from the field. *Zebrafish* 2007;1:21–40.
- Arunachalam M. Natural history of zebrafish (*Danio rerio*) in India. *Zebrafish* 2013;1:1–14.
- Suriyampola P, Shelton D, Shukla R, Roy T, Bhat A, Martins E. Zebrafish social behavior in the wild. *Zebrafish* 2016;1:1–8.
- Turnell E, Mann K, Rosenthal, Gerlach G. Mate choice in zebrafish (*Danio rerio*) analyzed with video-stimulus techniques. *Neurobiol Behav* 2003;2:225–226.

14. Spence R, Fatema M, Ellis S, Ahmed Z, Smith C. Diet, growth and recruitment of wild zebrafish in Bangladesh. *J Fish Biol* 2007;1:304–309.
15. Gerlach G, Lysiak N. Kin recognition and inbreeding avoidance in zebrafish, *Danio rerio*, is based on phenotype matching. *Anim Behav* 2006;6:1371–1377.
16. Spence R, Smith C. Mating preference of female Zebrafish, *Danio rerio*, in relation to male dominance. *Behav Ecol* 2006;5:779–783.
17. Lawrence C, Best J, James A, Maloney K. The effects of feeding frequency on growth and reproduction in zebrafish (*Danio rerio*). *Aquaculture* 2012;368:103–108.
18. Nasiadka A, Clark M. Zebrafish breeding in the laboratory environment. *Inst Lab Anim Res* 2012;2:161–168.
19. Kalueff A, Gebhardt M, Stewart A, Cachat J, Brimmer M, Chawla J, *et al.* Toward a comprehensive catalog of zebrafish behavior 1.0 and beyond. *Zebrafish Neuroscience Research Consortium (ZNRC)*. *Zebrafish* 2013;10:70–86.
20. Moretz J, Martins E, Robison B. Behavioral syndromes and the evolution of correlated behavior in zebrafish. *Behav Ecol* 2007;3:556–562.
21. Ramsay J, Feist G, Varga Z, Westerfield M, Kent M, Schreck C. Whole-body cortisol is an indicator of crowding stress in adult Zebrafish, *Danio rerio*. *Aquaculture* 2006;1–4:565–574.
22. Parker M, Millington M, Combe F, Brennan C. Housing conditions differentially affect physiological and behavioral stress responses of zebrafish, as well as the response to anxiolytics. *PLoS One* 2012;4:e34992.
23. Kaushik S, Georga I, Koumoundouros G. Growth and body composition of zebrafish (*Danio rerio*) larvae fed a compound feed from first feeding onward: toward implications on nutrient requirements. *Zebrafish* 2011;2:87–95.
24. Meinelt T, Schulz C, Wirth M, Kurzinger H, Steinberg C. Dietary fatty acid composition influences the fertilization rate of zebrafish (*Danio rerio* Hamilton-Buchanan). *J Appl Ichthyol* 1999;1:19–23.
25. Meinelt T, Schulz C, Wirth M, Kurzinger H, Steinberg C. Correlation of diets high in n-6 polyunsaturated fatty acids with high growth rate in zebrafish (*Danio rerio*). *Comp Med* 2000;1:43–45.
26. Geffroy B, Simon O. Effects of a spirulina platensis-based diet on zebrafish female reproductive performance and larval survival rate. *Cymbium* 2013;1–2:31–38.
27. Newman T, Jhinku N, Meier M, Horsfield J, Schausberger P. Dietary intake influences adult fertility and offspring fitness in zebrafish. *PLoS One* 2016;11:e0166394.
28. Martins S, Monteiro J, Vito M, Weintraub D, Almeida J, Certal A. Toward an integrated zebrafish health management program supporting cancer and neuroscience research. *Zebrafish* 2016;S1: S-47–S-55.
29. Barton C, Johnson E, Tanguay R. Facility design and health management program at the Sinnhuber Aquatic Research Laboratory. *Zebrafish* 2016;S1:S-39–S-43.
30. Farias M, Certal A. Different feeds and feeding regimens have an impact on zebrafish larval rearing and breeding performance. *SOJ Aquat Res* 2015;1:1–8.
31. Ulloa P, Iturra P, Neira R, Araneda C. Zebrafish as a model organism for nutrition and growth: towards comparative studies of nutritional genomics applied to aquacultured fishes. *Rev Fish Biol Fish* 2011;21:649–666.
32. Smith Jr., D, Barry R, Powell M, Nagy T, D'Abramo L, Watts S. Dietary protein source influence on body size and composition in growing zebrafish. *Zebrafish* 2013;3:439–446.
33. Tocher D, Agaba M, Hastings N, Bell J, Dick, J, Teale A. Nutritional regulation of hepatocyte fatty acid desaturation and polyunsaturated fatty acid composition in zebrafish (*Danio rerio*) and tilapia (*Oreochromis niloticus*). *Fish Physiol Biochem* 2001;4:309–320.
34. Nowosad J, Kucharczyk D, Katarzyna T. Enrichment of Zebrafish *Danio rerio* (Hamilton, 1822) diet with polyunsaturated fatty acids improves fecundity and larvae quality. *Zebrafish* 2017;4:364–370.
35. Izquierdo M, Fernandez-Palacios H, Tacon A. Effect of broodstock nutrition on reproductive performance of fish. *Aquaculture* 2001;1:25–42.
36. Jardine D, Litvak M. Direct yolk sac volume manipulation of zebrafish embryos and the relationship between offspring size and yolk sac volume. *J Fish Biol* 2003;2:388–397.
37. Uusi-Heikkilä S, Wolter C, Meinelt T, Arlinghaus R. Size-dependent reproductive success of wild zebrafish *Danio rerio* in the laboratory. *J Fish Biol* 2011;3:552–569.
38. Adatto I, Lawrence C, Thompson M, Zon L. A new system for the rapid collection of large numbers of developmentally staged zebrafish embryos. *PLoS One* 2011;6:1–7.
39. Matthews M, Varga Z. Anesthesia and euthanasia in zebrafish. *Inst Lab Anim Res* 2012;2:193–204.
40. Jobling M. Fish nutrition research: past, present and future. *Aquac Int* 2016;3:767–786.
41. Powell M, Pegues M, Szalai A, Ghanta V, D'Abramo L, Watts S. Effects of the dietary ω 3: ω 6 fatty acid ratio on body fat and inflammation in zebrafish (*Danio rerio*). *Comp Med* 2015;4:289–294.
42. Barton B, Schreck C, Fowler L. Fasting and diet content affect stress-induced changes in plasma glucose and cortisol in juvenile chinook salmon. *Progress Fish Culturist* 1988;1:16–22.
43. Schreck C, Contreras-Sanchez W, Fitzpatrick M. Effects of stress on fish reproduction, gamete quality, and progeny. *Aquaculture* 2001;1–4:3–24.
44. Campbell P, Pottinger T, and Sumpter J. Stress reduces the quality of gametes produced by rainbow trout. *Biol Reprod* 1992;6:1140–1150.

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