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Unsupplemented *Artemia* Diet Results in Reduced Growth and Jaw Dysmorphogenesis in Zebrafish

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1. Introduction

The number of laboratories using zebrafish as an experimental animal model has risen tremendously over the past two decades (Craig et al., 2006). As a result, the number of zebrafish facilities around the world has dramatically increased to meet the elevated demand for proper animal care and maintenance. In order to meet this demand, aquaculture facilities must employ husbandry protocols designed to produce a constant supply of healthy, viable eggs. Surprisingly, many husbandry strategies, particularly feeding protocols, are frequently passed down from members of one lab to another in a colloquial fashion without rigorous experimental validation. An ideal diet should consist of a minimal variety of foodstuffs designed to be nutritionally complete, simple to prepare, non-fouling, and cost-effective. Previous studies aimed at streamlining adult zebrafish feeding strategies in large aquaculture facilities have emphasized cost-effective, single-food models, but such diets lead to diminished survivorship and reproductive capacity (Goolish et al., 1999; Meinelt et al., 1999; Barnard & Bagatto, 2002), suggesting that these diets are lacking in some key nutritional component(s). Restricting adult fish diets to single foodstuffs, while desirable from a time and cost perspective, may not provide the trace mineral balance needed for adequate hormone and enzyme production, proper skeletal formation, and other biochemical or physiological needs. Nonetheless, given the intense breeding schedules many facilities are forced to adopt to meet research needs, a single component, high protein diet for enhanced egg production is frequently adopted.

Processed flake food diets are generally rich in proteins and essential trace elements required for normal physiological function. Despite their critical role in modulating somatic growth and basal metabolic rate in homeotherms (Eales & Brown, 1993), recent studies aimed at identifying alternative diets for improving the growth and reproductive capacity of zebrafish have paid little attention to minimum dietary iodine requirements. This may be due, at least in part, to the fact that zebrafish maintain a large reserve of T4 in the follicular colloid effectively allowing the animal to accommodate transient iodine deprivation (Brown et al., 2004). Additionally, iodized salts in system water and iodine-fortified processed

foodstuffs are commonly used to avoid iodine-deficiency pathologies such as hyperthyroidism and goiter. Nevertheless, environmental conditions such as poor water quality or malnutrition can lead to marked proliferation of thyroid tissue in some fishes (Hoover, 1984) indicating that care must be taken to maintain a minimum iodine level in artificial environments including zebrafish aquaculture facilities. Dietary supplements have been shown to prevent thyroid hyperplasia in the saltwater cyprinodont *Fundulus* (Pickford, 1954) and goiters in the Chinook salmon (Woodall & La Roche, 1964), but no study to date has effectively assessed the minimum iodine level for maintaining normal development and thyroid health in zebrafish.

While experimenting with varied diets in our facility, we observed that conditions of reduced iodine result in less robust somatic growth and an elevated incidence of jaw dysmorphogenesis. This prompted us to evaluate the developmental consequences of rearing zebrafish on a single live food or a mixed food diet with varying levels of iodine. Herein we demonstrate that a strict single-food *Artemia* diet, although high in the protein necessary for robust egg production, may also result in a morphological alteration of the zebrafish jaw architecture formed in response to swelling and hypervascularization of adjacent tissues.

2. Experimental methods

2.1 Animal housing and maintenance

Adult WIK wild-type zebrafish were raised, maintained, and spawned in the Zebrafish Model Organism Laboratory, located at the University of Cincinnati (UC) Genome Research Institute according to previously published protocols (Craig et al., 2006; Kimmel et al., 1995; Westerfield, 2000). Zebrafish fry were reared in a multi-rack system from Aquatic Habitats (Apopka, FL) with automated control of water chemistry (YSI Model 5200 control system, Yellow Springs, OH) utilizing a 14-h light/10-h dark cycle. Water chemistry was maintained at the following conditions to ensure optimal fish health: temperature: 28.5 ± 1 °C; pH: 7.5 ± 0.2; ammonia: 0 ± 0.25 mg/l; nitrite: 0 ± 0.20 mg/l; nitrate: 5 ± 5 mg/l; total hardness: 102 ± 17 mg/l; alkalinity 50 ± 17 mg/l. Conductivity was maintained at 750 ± 50 µS. Adult breeding stock fish were fed a standard diet of 3 day old HUFA-enriched (Super SELCO, Aquatic Ecosystems) *Artemia* in the morning plus a balanced flake food (Aquatox, Aquatic Ecosystems) in the afternoon.

2.2 Dietary paradigm

Fry were fed a mixed diet of *Paramecium*, rotifers, and Zeigler's larval diet (Zeigler Bros, Inc.) until they were capable of eating *Artemia* (approximately 1 month of age) and to ensure proper early development. Beginning at 1 month of age, fish were placed on either a live study diet of three *Artemia* feedings or a mixed control diet of two Aquatox flake and one *Artemia* feedings per day.

Dietary *Artemia* intake was measured by feeding *Artemia* to overnight fasted fish (17.2 mg per tank, 15 minutes feeding time to approach satiety). Fish were euthanized in concentrated 250 mg/l MS-222, eviscerated and the alimentary tract was removed. The gut was everted, and the total number of ingested shrimp counted under a stereomicroscope. Aquatox flake

food uptake was similarly measured by feeding fish to satiety (4.7 mg per tank, given 15 minutes feeding time) and then vacuuming up the excess food with a siphon, drying and weighing the uneaten excess.

2.3 Iodine analyses

Quantification of iodine in the foodstuffs was performed using ICP-MS as described previously for other heavy metals (Figueroa et al., 2008). Briefly, *Artemia* samples were cultured for 72 h, harvested and freeze-dried overnight in a lyophilizer (FreeZone 4.5 Liter Benchtop System, Labconco Corporation, Kansas City, MI). Three replicates of dried *Artemia* and system water samples (~30 mg) were placed in septum sealed glass tubes, treated with HNO_3 and microwave digested: power, 130 W; ramp, 1:00 min; hold, 1:00 min; temp, 75 °C. Subsequently, 0.1 mL of 30 % H_2O_2 was added and the samples were processed through the second digestion stage: power, 130 W; ramp, 1:00 min; hold, 1:00 min; temp, 110 °C. Samples were diluted with DDW to 10 mL and analyzed for total iodine content in continuous flow mode using an Agilent 7500ce ICP-MS (Agilent Technologies, Tokyo, Japan) equipped with a quadrupole mass analyzer.

2.4 Histology

Standard hematoxylin and eosin staining was performed at Oregon State University on both phenotypic and control juvenile zebrafish in order to ascertain the nature of the jaw dysmorphogenesis. Sagittal jaw sections were cut 4 micron thick, fixed in paraformaldehyde and stained with Alizarin Red according to previously published protocols (Cailliet et al., 1986). Adult fish were cleared and stained with Alizarin Red dye in order to visualize bone and pigment structure. Fish were fixed for 3 days in 10% phosphate buffered formalin. Fish were first rinsed in deionized water, bleached in 3% hydrogen peroxide / 2% potassium hydroxide to remove pigment, rinsed a second time in DI water, trypsin digested for eight days (3375 U/ml in 30% Sodium Borate) until a majority of the spine was visible, soaked in 0.5% KOH, soaked in Alizarin Red for several hours and destained in 2% KOH overnight. Stained fish were then transferred stepwise into increasing glycerol concentrations (90% final) for imaging and long-term storage.

2.5 Morphometrics

The effects of diet on normal fish growth were determined with standard growth metrics. Body mass measurements were made on anesthetized (125 mg/l MS-222) fish. Water accumulated on the body surface can result in significant overestimation of body mass and so it was removed by gently blotting the animal with absorbent paper (Kimwipe). Individual fish were then weighed three times on a 5-place analytical balance (Mettler-Toledo, Inc., Columbus, Ohio) and the average calculated. Total lengths (tip of the nose to the tip of the tail fin) were obtained from still images using a calibrated measurement tool in Rincon 7.1 software (Optronics Inc., Goleta, California) or using a manual (dial) caliper. Craniofacial development was similarly assessed from still images of Alizarin Red bone stained adult fish by measuring the lengths and widths of the Meckel's and ceratohyal complexes in fish exposed to the different dietary regimens.

2.6 Statistics

All statistics were performed in SigmaStat XI (Systat Software Inc., San Jose, California). Means were analyzed using Student two-tailed unpaired t-tests or nonparametric rank sum tests as needed. Probability (P) values of <0.05 were considered significant. Incidence levels were compared using a Yates-corrected Chi-square test.

3. Results and discussion

3.1 Dietary paradigm results in a 6-fold difference between *Artemia*-only and mixed food diets

Fish fed *Artemia* three times daily consumed an average of 1.2 mg of *Artemia* (51.6 mg wet weight) per feeding for a total of 3.6mg dry mass. Fish reared on the mixed food diet consumed a total of 4.8mg of dry mass per day (1.2mg *Artemia* twice and 2.37mg of Aquatox flakes once). Dietary and environmental iodine levels measured by ICP-MS (Table 1) indicated that the *Artemia* only diet contained just under one-sixth of the total iodine present in the mixed diet (11.48ng and 72.6ng, respectively).

Source	Concentration (ppm)
Crystal Sea Marine Mix[a]	0.12
Instant Ocean Sea Salt[a]	0.26
Seawater[b]	0.49-0.58
System Water (Instant Ocean + salts)	0.73 ± 0.26
SuperSelco Enriched *Artemia*[b]	3.19 ± 1.04
Aquatox Flakes[b]	27.38 ± 1.56

Table 1. Iodide concentrations of study-related salts, feed and supplements. Mean values are listed ± 1 s.d. [a](Bidwell and Spotte, 1985), [b]ICP-MS quantified.

The reduction in somatic growth may be the result of diminished endocrine output, possibly caused by an iodine or other nutrient deficiency (Ikeda et al., 1973). The smaller body size was not likely due to underfeeding since the fish were fed three satiating meals of *Artemia* daily. Further, there was no indication of impaired feeding capacity or precipitous wasting condition in grossly phenotypic fish, despite our expectation that the displacement of the lower jaw bones would negatively affect feeding ability. The *Artemia* fed fish were, in fact, visibly smaller throughout the study.

3.2 *Artemia*-only diet results in smaller, less sexually dimorphic individuals compared with mixed diets

The body mass and length of fish raised on the *Artemia* diet was significantly reduced compared with fish reared on a mixed diet (Table 2, P<0.0001) despite having made morphometric measurements well after the fish should have normally reached their terminal length (~150 dpf, unpublished data). Sexual dimorphism was clearly evident in the mixed diet population by 4 months of age, but the *Artemia* fed fish were only minimally dimorphic at 10 months of age. Interestingly, a diet of processed flake food alone also

	Artemia Only	Mixed Diet	P-value
Mass (mg)	139 ± 64 (78)	278.80 (15)	0.079
Length (mm)	25.4 ± 1.9 (78)	60.4 ± 2.1 (15)	<0.001
Meckle's Length (mm)	1.23 ± 0.29 (4)	1.52 ± 0.75 (2)	0.499
Ceratohyal Length (mm)	1.43 ± 0.53 (4)	1.79 ± 0.84 (2)	0.541
Iodine Content (mg/day)	0.11	0.28	n/a

Table 2. Body morphometrics of 160-180 day old fish on study diets. Mean values listed ± 1 s.d.

resulted in reduced terminal lengths when compared to fish fed *Artemia* only or mixed food diets (data not shown).

3.3 Thyroid enlargement leads to jaw bone displacement in fish fed low-iodine diets

The protruded mandibular jaw phenotype (Fig. 1) was observed within 1 week of initiating the single food *Artemia* diet, with the incidence level increasing logarithmically to 6.3% at 180dpf and reaching a plateau at 10.5% at 10 months of age (n=828 total fish). The incidence level in the population receiving the mixed diet was significantly lower throughout, reaching 3.4% at 10 months (n = 507 total fish, Yates-corrected Chi-square stat: 21.4, α = 0.05, 95% CI: 4.3 – 10.1%). A gradual increase in the extent of mandibular protrusion and degree of localized vascularization was observed throughout the 10 month test period with 78% of the phenotypic fish exhibiting large vascularized growths that protruded an average of 2.1% of their total body length (Fig. 1A). Histologic analyses revealed clear glandular involvement (Fig. 1B) with a distinct rotation of the basihyal jawbone from a sagittal to a more transverse orientation (Fig. 1C). The jaw dysmorphogenesis appeared to be the result of bone displacement by the enlarged thryroid adjacent to the basihyal none rather than a deficiency in osteogenesis, although the normalized Meckel's cartilage and ceratohyal bone lengths were marginally reduced compared to fish fed the mixed diet (Table 2, 19% and 20% reductions for Meckel's and ceratohyal lengths).
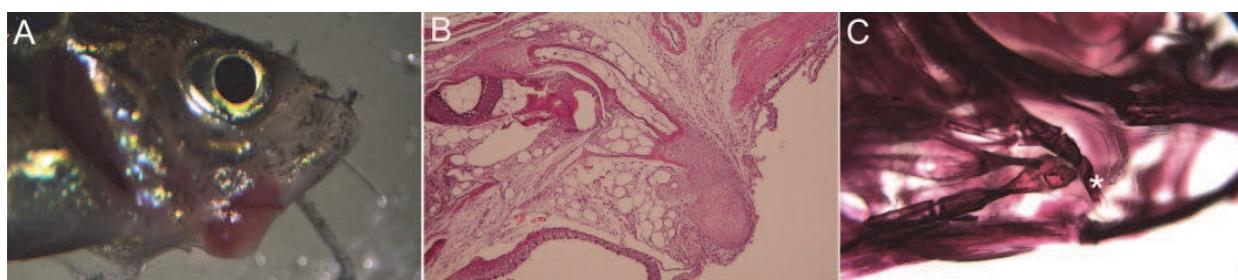

Fig. 1. Phenotypic characterization of low iodine diet-reared fish. Gross phenotype (A), histological section stained with hematoxilin and eosin (B), and Alizarin Red bone stain (C). Ventral rotation of the basihyal bone is indicated by an *.

The presence of an enlarged, hypervascularized tissue mass in proximity to the thyroid gland prompted us to speculate that the overall phenotype was due to an iodine deficiency. Because the teleost thyroid is an unencapsulated colloid-filled follicle (Gundernatsch, 1911), it is often difficult to classify the exact nature of piscine thyroid phenotypes. Nevertheless, several studies in other fish species have shown lower jaw deformities similar to those seen

in the *Artemia* fed zebrafish in this study (Gaylord & Marsh, 1912; Marine & Lenhart, 1911; Marine, 1914). The enlarged hypervascularized masses protruding beneath the jaw of New York hatchery salmon were attributed to thyroid carcinomas (Gaylord & Marsh, 1912). Brook trout in a Pennsylvania hatchery also appeared to have simple goiters (Marine, 1914) externally similar in appearance to the phenotype observed in our study. Histochemical analysis of fish reared on our low-iodine diet yielded no indication of cell over proliferation, nuclear atypia, or extra cellular layers typical of carcinomas, but instead showed evidence of pushing borders, organ involvement and hypervascularization typical of simple goiter (Gaylord & Marsh, 1912). Histological analysis also clearly indicated a ventrally-oriented bone displacement associated with the observed lower jaw protrusion, suggesting that the glandular tissue was impinging upon the jawbones and altering their normal orientation. Although our studies terminated after six months, chronic iodine deprivation would have eventually led to 100% incidence of thyroid malformations, however the gradual hyper-vascularization appeared to exhibit an endpoint at 10.5% of the study fish. Other contributing factors such as environmental stresses and individual fish health may also have modulated the observed phenotypes.

4. Conclusion

Freshwater fish generally require at least 1 – 4 mg total I kg^{-1} (Watanabe et al., 1997) and a dietary minimum of 2.8 mg I kg^{-1} (Lovell, 1979), but plasma iodide levels in freshwater fish vary greatly indicating a wide spectrum in the efficiency of iodine uptake and its use. Given the low 0.1 – 10 µg/l iodine content of freshwater (Eales, 1979) and a metabolic need not met by many natural or artificial environments (Schlumberger, 1954), these fish generally rely more heavily on dietary sources of iodine than do marine fish. Freshwater fish obtain iodine from food by transport through the gut, environmental uptake across the gills, and a very small portion through hormone recycling. Our data suggests that the minimum iodine level required to prevent diminished growth and other apparent iodine-related phenotypes in zebrafish is somewhat lower than for other freshwater fish, falling between the iodine content in our *Artemia* and mixed food diets (being in the range of 0.11 to 0.28 mg I kg^{-1} per day above the negligible 0.7 ppm environmental exposure). It remains unclear whether iodine supplementation in excess of the mixed diet level (e.g. two *Aquatox* flake feedings per day or *Artemia* supplemented with Kent's iodine) would correct the observed gland enlargement and bone displacement, but previous studies have shown that trout goiters can be attenuated with a potassium iodide supplement in as little as 10 days of exposure time (Marine & Lenhart, 1911; Marine, 1914).

We support the notion that the minimal costs associated with adding iodine-rich flake food to the diet of these fish both is well worth the effort as it results in improved fish health and reduces the time needed to reach sexual maturity.

5. Acknowledgment

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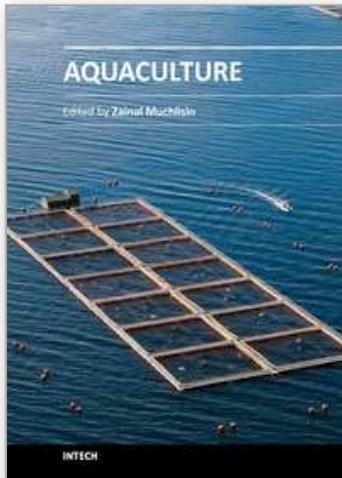
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This book provides an understanding on a large variety of aquaculture related topics. The book is organized in four sections. The first section discusses fish nutrition second section is considers the application of genetic in aquaculture; section three takes a look at current techniques for controlling lipid oxidation and melanosis in Aquaculture products. The last section is focused on culture techniques and management, ,which is the larger part of the book. The book chapters are written by leading experts in their respective areas. Therefore, I am quite confident that this book will be equally useful for students and professionals in aquaculture and biotechnology.

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