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Evaluation of *Moringa oleifera* as a dietary supplement on growth and reproductive performance in zebrafish

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Abstract

The leaves of the *Moringa oleifera* (*Moringa*) tree contain a significant source of protein, vitamins and minerals, and are considered as an important dietary supplement in countries where chronic malnourishment is linked to poor fetal development. We evaluated the effectiveness of the *Moringa* leaf as a supplemental replacement for vitamins, minerals, and protein in a formulated zebrafish diet and the impact that it may have on growth and reproductive outcome. Diets included a formulated control (FC) containing an array of vitamins and mineral supplements (pre-mixes), dried ground *Moringa* only (M), formulated control minus vitamin and mineral pre-mixes (Fvm), and formulated control minus vitamin and mineral pre-mixes and supplemented with *Moringa* (FM). Juvenile zebrafish were fed experimental diets *ad libitum*. After a 12 week feeding period, each treatment group was evaluated based on growth and reproductive performance. The M treatment showed the least growth performance (length and weight gain) and no reproductive success (no egg production). Although small, M fish appeared otherwise healthy, with survivorship at ca. 70%, suggesting, *Moringa* can serve as a single ingredient source for a short period of time. FC showed the highest growth performance, and had the highest reproductive success. Growth performance and reproduction in the Fvm diet was greatly reduced. However, inclusion of *Moringa* (FM) promoted significant, but not total, recovery of growth and reproductive metrics. These data suggest that *Moringa* leaves can serve as an acceptable supplement for macro and micronutrients in the diet and could, in part, reduce problems associated with nutrient deficiencies.

Keywords

Moringa oleifera; zebrafish; nutrition; reproduction; dietary supplement

Introduction

Malnutrition has been linked to a number of developmental abnormalities observed from birth to adulthood. The mechanisms involving malnourishment begins *in utero* and could continue throughout the life of an individual (1). Because of this, research remains ongoing in an effort to eliminate developmental deficits linked to poor nutrition especially in

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Conflicts of Interest

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developing countries where malnutrition is prevalent. Local plant source nutrients can, in many cases, provide adequate amounts of vitamins, minerals, protein and energy in the diet. Supplementing diets with vitamins and minerals from sustainable regional plant sources is critical in areas where intensive agriculture and production animals are limited due to socioeconomic or short-term climatic (environmental) challenges. Importantly, the lack of bioavailable nutrients is known to largely affect human development from birth to adulthood (1). In animals, deficiencies in vitamin A, E, iron, and certain minerals have been shown to have a profound impact on embryonic development. In fact, studies have shown that lack of certain minerals, vitamins, and nutrients in the diet leads to significant skeletal pathologies in animal models including zebrafish (2). Likewise, diets deficient in vitamin E and fatty acids can affect the fertilization and mortality in zebrafish (3). Recently, zebrafish have emerged as a powerful model system to study nutrition and dietary factors related to human health and disease. Previous studies have assessed the differences between defined and undefined diets as well as live and natural sources in the diet on spawning, reproductive success and body composition in the zebrafish (4, 5, 6, and 7). Moreover, researchers are investigating the significance of certain nutrients in the diet and have shown that dietary fatty acids can impact fertilization in zebrafish (8). Overall, those studies show that proper accumulation, absorption, and maintenance of certain nutrients are vital for healthy embryonic development.

In the tree *Moringa oleifera*, an array of vitamins and minerals are abundantly stored (9). Found endemically in India but cultured widely, recent reports have suggested dietary supplementation with leaves from *Moringa oleifera* (*Moringa*) could significantly improve the quality of embryonic health and development (10) in human health and nutrition. *Moringa* contains an abundance of macro and micronutrients as well as antifungal, anti-inflammatory and antioxidant factors (11, 12). Also, *Moringa* has become well known for its medicinal properties involved in reducing hypertension, cancer, diabetes, cardiovascular disease, and hypercholesterolemia (13, 14, 15, and 16); and is currently used as a food source in countries lacking essential dietary nutrients (17). Animal studies have shown that the supplementation of *Moringa* in the diet as a source of vitamins and minerals can improve the quality of reproduction and overall growth performance (18, 19).

To date, few studies have investigated plant-based nutrients particularly *Moringa* on overall growth outcomes in fish (20, 21). In these studies, growth performance and digestibility of fish fed a *Moringa*-based diet were determined. However, these studies did not examine the effects of *Moringa* on fecundity and embryonic development. Using the zebrafish model, our goal is to understand the effect of *Moringa* supplementation, or as a sole source ingredient, on embryonic development by evaluating growth and reproductive performance in zebrafish.

Materials and Methods

Zebrafish care and maintenance

Adult zebrafish (*Danio rerio*) AB strain were housed in the Aquatic Animal Research Core at UAB. Breeding populations were held at approximately 28°C on a 14-h light/ 10-h dark cycle. All fish were maintained at a 5 individuals per L as described in Smith et al. (2013). Embryos were collected randomly from a mass spawn of adult zebrafish (fed previously the

FC diet described below). At 5 days post fertilization (dpf), hatched larvae were fed *ad libitum* the rotifer *Brachionus plicatilis* enriched with *Nannochloropsis* (RotiGrow ® Omega, Reed Mariculture) three times daily until day 28. At day 28 fish were randomly distributed into 1.8 L tanks representing one of four diet treatments (n = 12 individuals per tank, 4 tanks per diet treatment). A photograph of all fish in each tank was recorded with a Nikon DS FIL or Nikon D-70 and the length of the fish was determined by NIS Elements 3.1 image analysis from a digital image. Each fish was measured for total length (from the tip of the snout to the longest caudal fin ray) using the software's ruler function. Average lengths were evaluated by ANOVA to insure no significant differences among the treatments at the beginning of the experiment. All zebrafish were maintained at 28°C and 1500 uS/cm conductivity in a recirculating system (Aquaneering, Inc. San Diego, CA). Flow rates were adjusted to provide at least two water changes per hour within each tank. Municipal tap water was filtered through 5 µm sediment filter, followed by charcoal, R/O, and a cation/anion exchange resin (Kent Marine, Franklin, WI) prior to the addition of synthetic sea salts (Instant Ocean) to obtain final conductivity for the system water source. Tanks were maintained on the same conditioned water system throughout the experiment, but rotated across rack positions at weekly intervals to reduce environmental confounding from noise, light, vibration, or other sources. Tanks were siphoned weekly to remove any uneaten feed or debris. Sodium bicarbonate was used to maintain pH of the system water (adjusted daily to maintain approximately 7.4 pH). At least 20% of system water was exchanged weekly. Total ammonia nitrogen, nitrite, and nitrate were measured colorimetrically (Mars Fish Care, Inc., Chalfont, PA).

Feed Formulations

Each diet was produced with a single, common base mix (all ingredients minus the supplemental vitamins and minerals) using chemically defined ingredients (see Table I). The base mix was divided and the other respective ingredients were added to produce the four formulated feeds. The control diet (FC) is supplemented with vitamin and mineral premixes, as well as potassium phosphate and ascorbypalmitate. The Fvm diet contained all ingredients and macronutrients associated with the base mix, but no vitamins or minerals (as premixes) were supplemented (replaced by mass with diatomaceous earth. The FM diet had all macronutrients found in the FC and Fvm diets; however, powdered Moringa leaves (obtained from The World Vegetable Center (AVRDC) in Taiwan) were added by mass as a replacement for the vitamin and mineral premixes. An additional diet was solely comprised of the powdered Moringa leaves (M). Diets were mixed in an orbital mixer (Kitchen Aid) and then extruded with a Kitchen Aid extruder (KPEXTA) and were air-dried to ~10% moisture content before storage. Extruded pellets were ground to a powder (250–500 µm sieved).

Feeding Trials

Trial 1—At 28 dpf (days post fertilization), the fish tanks were divided into four treatments (4 replicate tanks per treatment). Each treatment was assigned to one of four diets described previously. Fish were fed *ad libitum* three times daily (at a rate exceeding 5% of body weight per day) their respective diets for 21 weeks. Starting at week 12, randomly chosen males and females were combined in breeding tanks (4 breeding pairs/treatment/week) and

reproductive success was assessed (see below) once each week for 9 weeks (36 breeding events for each treatment). At 21 weeks all fish were weighed and photographed individually. Fish were returned to their respective tanks and all experimental treatments were subsequently fed the control diet for an additional 5 weeks (from week 21 to week 26). At 26 weeks reproductive success was again determined for 5 additional weeks (from week 26 to week 31) while all treatments remained on the control diet (2 breeding pairs/treatment/week except Moringa, which had 1 breeding pair per week). At the end of the 31 week feeding period, fish were euthanized according to IACUC protocols by placing in an ice water bath. Zebrafish were sexed by the examination of genital papillae or external gonadal evaluation, and the averages of terminal weights and lengths were compared between males and females for each treatment.

Reproductive performance

When reproduction was assessed during the 31-week feeding trial, two females and one male were placed overnight in a breeding chamber (Aquaneering) and bred per treatment group once weekly. Embryos were collected within one hour after spawning and counted for total number produced. Afterwards, the total number of embryos per feed group were transferred to embryo medium and stored in an incubator at 28°C overnight. The following day, embryos were re-counted at approximately 24–30 hpf (hours past fertilization) for viability. Viability was determined by assessing the embryo at its appropriate stage of development (23). Embryos were considered viable as long as they appeared to be developing whether or not development was delayed.

Trial 2—Additional embryos were collected from broodstock and reared to 28 dpf as described in Trial 1. At day 28 fish were randomly distributed into 1.8 L tanks representing one of three diet treatments (n = 12 individuals per tank, 4 tanks per diet treatment). All fish in each tank were photographed with a Nikon DS FIL or Nikon D-70 and the length of each fish was determined by NIS Elements 3.1 image analysis. Each fish was measured for total length (from the tip of the snout to the longest caudal fin ray) using the measurements tool/function embedded in the software. Average lengths were evaluated by ANOVA to insure no significant differences among the treatments at the beginning of the experiment. Individuals were fed one of three diets, representing (1) the FC control, fed ad libitum three times daily for 8 weeks, (2) fed ground Moringa leaves only for 4 weeks, then switched to the FC diets for 4 additional weeks, or (3) fed ground Moringa leaves only for 8 weeks. Weight and length were recorded each week during the 8 week period. The body condition index (k) was calculated for males and females from each treatment using the following formula: $K = ((\text{mean weight} * 100) / (\text{mean length}^3))$

Statistical Analysis

Differences between treatments for terminal weights and lengths, mean weekly egg production, and body condition index were determined by 1-way ANOVA, with subsequent post hoc comparisons (Tukey's HSD) to identify treatment differences. Data for terminal weights, terminal lengths, survivorship, mean weekly egg production, and body condition index were analyzed by R statistical software. Differences in survivorship for Trial 1 were

first analyzed by a 2×4 contingency table and Chi-Square analysis to determine if there was there were any significant differences in survivorship.

Pairwise differences between treatments were then analyzed by 2×2 contingency tables and Chi-Square analysis to determine which treatments were significantly different. For trial 2, data and analyses were separated by sex, to account for weight differences occurring between males and females. In Trial 2, linear regression analysis was used to assess differences in weight gain between the control and M-C treatments (24). The slopes were determined to be significantly different if $p < 0.05$. Values of $p < 0.05$ were considered statistically significant, and are indicated in the graph by an A-B-C designation. Comparisons among males are designated with capital letters, and comparisons among females are designated using lower case letters. If the letters are different, this indicates statistical significance.

Results

Trial 1 - Growth and Survival

After 21 weeks, zebrafish fed the Fvm diet were significantly smaller than the control but were bigger than the *Moringa* only fed fish (Figure 1 A, B). Similarly, fish fed the FM diet had a higher mass than both M and Fvm fed groups, but smaller than the control group. Fish fed *Moringa* only were smaller compared to fish fed the other formulated diets. In addition, at the end of the 21-week feeding trial survival was similar for the FC, Fvm and FM treatments (97, 96, and 89 %, respectively), and significantly higher than those fed *Moringa* only (71% survival).

Fecundity and embryo viability

Mean weekly egg production was highest in the control diet, and was significantly higher than the Fvm diet, but not significantly different from the FM diet (Figure 2A). The total number of embryos produced from weekly spawning events over nine weeks (Figure 2B) was highest in those fed the control diet and reduced in those fed Fvm or FM. Fish fed *Moringa* only did not produce eggs. In addition, fish fed the Fvm and FM diet also had the lowest embryo viability, 41 and 50%, respectively, as compared to the control (74 %) (Figure 2 B).

Zebrafish remaining in the trial were all fed the control diet for 10 additional weeks. At 31 weeks there was no significant difference in wet weights among the control and experimental diets (Figure 3). Mean weekly egg production did not differ significantly among the control and experimental diets (Figure 4A). The total number of embryos produced from weekly spawning events over five weeks (Figure 4B) was similar among those fed control diets and those fed the *Moringa* only or FM diets for 21 weeks and then switched to the control diets for 10 weeks. For those fed previously the Fvm diet, total embryos numbers were reduced. Embryo viability was similar among all treatments, ranging from 76 to 97 % (Figure 4B)

Trial 2 – Growth

Mean wet weight increased from 48 to 290 mg in zebrafish fed the control formulated diets for 8 weeks (Figure 5A). Fish fed Moringa only and then switched to the formulated diet after week four showed a significant increase in weight and length within one week (Figure 5A, B). Regression analysis of the wet weight comparing similar sized zebrafish indicated that zebrafish switched from Moringa to the formulated diet had higher growth rates ($df = 6$; $P = 0.027$) than those fed the control formulated diet. At week eight, females had higher weights than males in all diet treatments (Figure 6A). Mean terminal weight and body length were smallest in the M treatment (Figure 6A, B). Mean body condition indices (K) were the same between males and between females in zebrafish fed the control formulated diet and those switched from Moringa to the formulated diet, but were lower in those fed Moringa only (Figure 6C).

Discussion

The evaluation of *Moringa oleifera* supplementation on growth or reproductive performance has been reported in a variety of animals including rabbits, cows, goats, mice, and some species of fish (18, 19). Those studies infer that the supplementation of *Moringa oleifera* into the daily diet can enhance growth and reproductive outcomes in animals. In this study, we evaluated the impact of *Moringa oleifera* as a replacement for a suite of dietary vitamins and minerals and as a short-term sole source of nutrition on both growth and reproductive performances in the zebrafish.

It is important to note that, in the absence of supplemental vitamins and minerals in the formulated diet, zebrafish do not grow and reproduce to the extent observed in zebrafish fed supplemental vitamins and minerals. The current study cannot identify which specific micronutrient (s) promote survival, growth and reproductive performance. However, this is one of the first studies to suggest that micronutrient availability is important in zebrafish diets. Evaluations of growth and reproductive performances show that dried *Moringa leaves* will presumably serve as a partial replacement for these vitamin and mineral supplements, resulting in partial recovery of growth and reproductive performances. In fact, those individuals that did not receive vitamins/mineral or Moringa did not recover reproductive performance when fed a normal diet, suggesting the lack of adequate micronutrient nutrition during juvenile development is essential to future reproductive success.

When fed as a sole source of nutrition, survivorship, growth and reproductive success of zebrafish all decreased; however, the remaining fish, although small, did not show morphological anomalies sometimes associated with poor nutrition. In fact, when zebrafish fed Moringa only for a short term were returned to a complete diet they recovered in growth and reproductive performances within weeks. Consequently, we suggest that the nutritional quality of *Moringa* alone is sufficient to sustain viability in a significant segment of the population.

Conclusion

Our evaluation further supports data that show the nutritive content of *Moringa* is sufficient to maintain and/or enhance the quality of reproductive and overall health in zebrafish. Future studies would involve our understanding of the specific micro- and macro-nutrients within *Moringa* that play a role in enhancing growth and reproduction performances. Previous studies have reported that diets deficient in vitamin E can affect zebrafish viability and function (3). In addition, reports have shown that diets lacking FA affects fecundity in zebrafish (8). Therefore, we suggest that the availability of these and other nutrients in *Moringa* contributes to the overall growth and reproductive outcomes examined in the zebrafish.

It may be useful to translate these findings to other animals or even human populations. We suggest that, in the absence of sufficient dietary macronutrients and micronutrients, *Moringa* can serve as a short-term substitute for a suite of nutrients. Consequently, when food is limited in populations exposed to acute food shortages (associated with famine in response to environmental impacts or natural disasters), *Moringa* can serve as a nutrient rich food source. This would appear to be of particular importance to juveniles within a population who require adequate nutrition during that period of development. As a short term nutritional intervention, *Moringa oleifera* shows great promise.

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Figure 1. Comparison of **A)** wet body weight and **B)** total body length between feed treatments at week 21. Data are expressed as means $\pm$ SE. If the letters over the columns are different, the treatments differ significantly ($p < 0.05$). FC = formulated control; M = dried, ground *Moringa* leaves; Fvm = FC minus vitamin and mineral mix; FM = FC minus vitamin and mineral mix, supplemented with *Moringa*.

Figure 2.

A. Box and whisker plot depicting embryos produced per breeding pair per week for each treatment during weeks 13–21. Mean values are included as diamonds, and the line through the box represents the median. The bottom and top of the box represent the first and third quartiles, respectively. Minimum and maximum values are shown with whiskers. Designation for significance is same as previously described in Figure 1. FC = formulated control; Fvm = FC minus vitamin and mineral mix; FM = FC minus vitamin and mineral

mix, supplemented with *Moringa*. The M treatment (dried, ground *Moringa* leaves) exhibited no reproductive success during weeks 13–21, and is not shown in the graph.

B. Total embryo production and percent viability for each treatment during weeks 13–21. The columns in black represent total embryos produced during weeks 12–17, while the columns in white represent the percentage of total embryos produced that were viable.

Figure 3.

Comparison of wet weights at week 31. Prior to week 31, all treatments were fed the control diet for a ten week period and then assessed for recovery. Data are expressed as means $\pm$ SE. Designation for significance is same as previously described in Figure 1. FC = formulated control; M = dried, ground *Moringa* leaves; Fvm = FC minus vitamin and mineral mix; FM = FC minus vitamin and mineral mix, supplemented with *Moringa*.

A

B

Figure 4.

A. Box and whisker plot depicting embryos produced per breeding pair per week for each treatment during weeks 26–31. Mean values are included as diamonds, and the line through the box represents the median. The bottom and top of the box represent the first and third quartiles, respectively. Minimum and maximum values are shown with whiskers.

Designation for significance is same as previously described in Figure 1. FC = formulated control; M = dried, ground *Moringa* leaves; Fvm = FC minus vitamin and mineral mix; FM = FC minus vitamin and mineral mix, supplemented with *Moringa*.

B. Total embryo production and percent viability for each treatment during weeks 22–25. The columns in black are represented by total embryos produced, while the columns in white represent the percentage of total embryos produced that were viable.

Figure 5.

Growth curves for **A**) wet body weight and **B**) Total body length for each feed treatment. Data are expressed as means $\pm$ SE (n=36 fish per treatment). FC = formulated control; M = *Moringa* only; M-C = *Moringa* fed for first four weeks, then FC fed for remaining four weeks.

A

B

Figure 6.

Comparison of **A**) Terminal wet weight and **B**) Terminal total body length for males and females from each treatment at week 8. Data are expressed as means $\pm$ SE (n=36 fish per treatment). Designation for significance is the same as previously described in Figure 1, with the exception that uppercase letters denote significance between males, while lowercase letters denote significance between females. FC=formulated control; M= *Moringa* only; M-C= *Moringa* fed for first four weeks, then FC fed for remaining four weeks.

C. Body condition index (K) for males and females from each treatment at week 8. Data are expressed as means $\pm$ SE. FC=formulated control; M= *Moringa* only; M-C= *Moringa* fed for first four weeks, then FC fed for remaining four weeks. The designation for significance is same as described previously in Figure 1. The uppercase letters represent differences between males, while lowercase letters represent differences between females.

Table I

Itemization of feed formulations

Ingredient	FC	Fvm	FM	M
casein-vitafree	25.00	25.00	25.00	0
fish protein hydrolysate	20.00	20.00	20.00	0
wheat gluten	10.00	10.00	10.00	0
soy protein isolate	5.00	5.00	5.00	0
dextrin	12.85	12.85	12.85	0
alpha cellulose	0	4.00	0	0
diatomaceous earth	0	4.19	0	0
sodium alginate	5.38	5.38	5.38	0
menhaden oil	4.67	4.67	4.67	0
soy lecithin refined	4.00	4.00	4.00	0
vitamin premix	4.00	0	0	0
mineral premix	3.00	0	0	0
corn oil	2.33	2.33	2.33	0
canthaxanthin 10%	2.31	2.31	2.31	0
potassium phosphate monobasic	1.15	0	0	0
betaine	0.15	0.15	0.15	0
cholesterol	0.12	0.12	0.12	0
ascorbypalmitate	0.04	0	0	0
<i>Moringa oleifera</i>	0	0	8.19	100.00
Total Percent Ingredient	100.00	100.00	100.00	100.00