Both Dietary Ratio of n–6 to n–3 Fatty Acids and Total Dietary Lipid Are Positively Associated with Adiposity and Reproductive Health in Zebrafish

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ABSTRACT

Background: Controversial findings have been reported in human and animal studies regarding the influence of n–6 (ω-6) to n–3 (ω-3) fatty acid ratios on obesity and health. Two confounding factors may be related to interactions with other dietary lipid components or sex-specific differences in fatty acid metabolism.

Objective: This study investigated main and interactive effects of total dietary lipid, ratio of n–6 to n–3 fatty acids, and sex on growth, adiposity, and reproductive health in wild-type zebrafish.

Methods: Male and female zebrafish (3 wk old) were fed 9 diets consisting of 3 ratios of n–6 to n–3 fatty acids (1.4:1, 5:1, and 9.5:1) varied within 3 total lipid amounts (80, 110, and 140 g/kg) for 16 wk. Data were then collected on growth, body composition (determined by chemical carcass analysis), and female reproductive success (n = 32 breeding events/diet over 4 wk). Main and interactive effects of dietary lipid and sex were evaluated with regression methods. Significant differences within each dietary lipid component were relative to the intercept/reference group (80 g/kg and 1:4:1 ratio).

Results: Dietary lipid and sex interacted in their effects on body weight (P = 0.015), total body length (P = 0.003), and total lipid mass (P = 0.029); thus, these analyses were stratified by sex. Female spawning success decreased as dietary total lipid and fatty acid ratio increased (P = 0.030 and P = 0.026, respectively). While total egg production was not associated with either dietary lipid component, females fed the 5:1 ratio produced higher proportions of viable embryos compared with the 1:4:1 ratio (median (95% CI): 0.915 (0.863, 0.956) vs 0.819 (0.716, 0.876); P < 0.001).

Conclusions: Further characterization of dietary lipid requirements will help define healthy balances of dietary lipid, while the sex-specific responses to dietary lipid identified in this study may partially explain sex disparities in the development of obesity and its comorbidities. Curri Nutr 2020;4:nzaa034.

Keywords: zebrafish, diet-induced obesity, dietary lipid composition, reproductive health, body composition

Original Research

Introduction

Defining the effects of dietary lipid on adiposity and human health remains a fundamental challenge. Conflicting results from studies evaluating the effects of diets with high lipid content on weight gain and metabolism have led to multiple debates regarding the magnitude to which dietary lipid contributes to obesity. Recent publications have noted that different responses to dietary lipid may be attributed to variations in fatty acid composition profiles, suggesting that lipid quality may be equally important as lipid quantity in the influence of obesity development and metabolic syndrome (1–3). Individual fatty acids can vary significantly in oxidation and deposition rates, which may, in turn, influence outcomes in weight gain, adiposity, and health in both human and animal models (1,2,4–6).

In mammals and fish, n–6 and n–3 fatty acids are crucial for nutritional health, physiology, and reproduction (7). They are categorized as essential and must be obtained through diet (8). Several studies have proposed that imbalances in the ratio of n–6 to n–3 fatty acids can neg-
atively impact health. Empirical and epidemiological data suggest that an excess intake of n-6 fatty acids increases inflammation in metabolic tissues (9–12); however, results from other studies indicate that arachidonic acid (ARA; 20:4n–6) also serves as a precursor for a group of potent anti-inflammatory molecules. Significant associations of increased dietary n-6 fatty acid content with both decreased inflammation and increases in lean tissue mass have also been observed (13–15). In addition to metabolic health, fertility and reproductive performance have also been linked to the ratio of n-6 to n-3 fatty acids in multiple species (16–20). As previous research has demonstrated that n-6 and n-3 fatty acid intake can significantly impact physiology and disease, a better understanding is needed regarding whether a specific ratio of n-6 to n-3 fatty acids is required for optimal health, and whether the responses of these ratios on health are modified by the total amount of dietary fat (21).

Sexual dimorphisms in the response to dietary manipulation must also be considered for obesity-related outcomes. Males and females differ in terms of how and where body fat is stored, hormone secretion in response to fat, and how the brain responds to signals that regulate body fat (22, 23). While sex-specific responses to dietary manipulation have been observed in multiple species, the influence of sex on the association between obesity-related phenotypes and dietary lipid composition has not been well defined (1, 24, 25). Therefore, when studying the impacts of dietary lipid manipulation on obesity, any potential sexually dimorphic responses should be considered (24).

To address gaps in our knowledge regarding the impacts of dietary lipid composition on obesity and metabolic health, many researchers have turned to animal models to answer these questions. In recent years, results from multiple studies have demonstrated that zebrafish are a powerful model for diet-induced obesity in humans and also offer multiple advantages over rodent models (26–31). Similar to humans, zebrafish exhibit increased weight gain, adiposity, and serum triglycerides (TGs) when fed a high-fat diet (HFD), as well as early evidence of metabolic diseases (26, 30). In this study, we examined both the individual and combined effects of total dietary lipid and dietary ratios of n-6 to n-3 fatty acids on weight gain, body composition, and reproductive success in juvenile male and female wild-type zebrafish. Results from this study will further define dietary lipid requirements for zebrafish in a research setting and translate this information to studies in human nutrition and health.

Methods

Diet preparation

Nine chemically defined experimental diets were formulated and produced using purified and semipurified ingredients (Supplemental Table 1). To ensure that all other macronutrients remained constant among diets, experimental diets were formulated with a single, common base mix. Total dietary lipid amounts were adjusted with AlphaCell™, a nonnutritive bulking agent (MP Biomedicals, LLC). Safflower oil (food grade; MP Biomedicals, LLC) served as the primary source of n-6 fatty acids, while menhaden fish oil (Virginia Prime® Gold Fish Oil; Omega Protein, Inc.) supplied the primary source of n-3 fatty acids. The amounts of each oil were adjusted to achieve the desired amount of total lipid and n-6 to n-3 fatty acid ratio for each diet. Diet analysis for crude fat and fatty acid composition was performed by Eurofins Scientific Laboratories, Inc. (Supplemental Table 2). Crude fat was determined by ethyl ether extraction, while fatty acid composition was determined by GC according to the American Oil Chemists’ Society methods Ce2–66 and Ce1–62 (32, 33). Analyses were performed for the 4 diets considered the “extremes” as a cost-saving measure, and values for other diets were interpolated.

The ingredients for the base mix were combined first using a KitchenAid Professional 600 Series Orbital Mixer (Whirlpool, Inc.). Next, the safflower and menhaden oils were added to each diet using a Cuisinart Food Processor (Conair, Inc.). Diets were then extruded with a KitchenAid Extruder (KPEXTA; Whirlpool, Inc.) fitted with the pastamaker attachment. Feed strands were air-dried on wire trays for 24 h and then stored at 4°C in air-tight storage bags until use. Prior to feeding, diets were ground to a powder (250–500 μm, sieved).

Experimental protocols

This study was conducted using recommendations of the Guide for the Care and Use of Laboratory Animals, NRC (34). All procedures abided by standard requirements for husbandry and euthanasia (34, 35). Protocols were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham (UAB).

Zebrafish were housed in 4 re-circulating systems with mechanical, chemical, and biological filtration and UV sterilization (Aquanerding, Inc.) on a 14-h light/10-h dark schedule. Tanks were siphoned weekly to remove uneaten food/debris, and flow rates were adjusted to provide a minimum of 2 water changes/h per tank. Sodium bicarbonate was added to adjust pH to the desired level as needed. At minimum, 20% of conditioned system water was exchanged once weekly from each system. Prior to being added to the re-circulating systems, municipal tap water was filtered through a reverse osmosis unit (Kent Marine) and conductivity was adjusted with synthetic sea salts (Instant Oceant). Water-quality parameters (given in Table 1 and Supplemental Table 3) were maintained at recommended levels (35) in all re-circulating systems throughout the experiment.

Zebrafish embryos (AB strain) were acquired from the Zebrafish Research facility at UAB. Due to the large sample size required for the study, zebrafish were divided into 4 cohorts and stocked once weekly over a 4-wk period. Embryos were collected, maintained until 5 d post-fertilization (dpf), and then polycultured with the rotifer Brachionus plicatilis in 6-L static tanks (240 larvae/tank), as previously described (36). Beginning at 11 dpf, each tank of fish was fed 25 mL of concentrated stage I Artemia nauplii (>500 nauplii per fish) 3 times daily. At 22 dpf, all fish were combined and 56 fish were randomly sampled to obtain initial wet weights. The remaining fish were randomly distributed into 2.8-L tanks at a density of 14 fish/tank (36 tanks/cohorts/144 tanks total for the study). After stocking, tanks of fish were randomly assigned to 1 of 9 diets (n = 16 tanks and 224 fish total per diet; within each cohort, n = 4 tanks and 56 fish per diet) and haphazardly, but evenly, distributed across rack positions on all 4 re-circulating systems. Each system held 4 tanks/diet, or 36 tanks/total.

During the 16-wk feeding trial (initiated at 23 dpf), each tank of fish was fed a daily ration of ~5–7% of body weight, with half the ration fed at ~0900 h and the other half at ~1700 h. Fish weights from each diet were monitored weekly to maintain this ration throughout the feeding trial. Using Excel’s RANDBETWEEN function, 25% of fish tanks from
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TABLE 1  Experimental water quality conditions for each recirculating system

| Parameter          | Target range       | Recirculating system |
|--------------------|--------------------|----------------------|
| pH                 | 6.80–8.50          | 7.45 ± 0.01          | 7.40 ± 0.01 | 7.39 ± 0.01 | 7.40 ± 0.01 |
| Conductivity, mS/cm| 1.40–1.50          | 1.42 ± 0.01          | 1.50 ± 0.01 | 1.52 ± 0.01 | 1.49 ± 0.01 |
| Salinity, ppt      | 0.60–0.70          | 0.69 ± 0.003         | 0.70 ± 0.003| 0.70 ± 0.004| 0.70 ± 0.003|
| Temperature, °C    | 27–28              | 27.4 ± 0.02          | 27.2 ± 0.02 | 27.2 ± 0.02 | 27.9 ± 0.03 |
| TAN, mg/L          | ~0                 | 0.01 ± 0.01          | 0.03 ± 0.03 | 0.01 ± 0.01 | 0.01 ± 0.01 |
| Nitrite, mg/L      | ~0                 | 0.19 ± 0.08          | 0.33 ± 0.09 | 0.36 ± 0.07 | 0.22 ± 0.08 |
| Nitrate, mg/L      | <200               | 101 ± 1.25           | 100 ± 0.0   | 100 ± 0.0   | 100 ± 0.0   |
| Alkalinity, mg/L CaCO₃ | 50–150           | 50–52                | 50–52       | 50–52       | 50–52       |

Values are mean ± SEM obtained over the course of the experiment. mS, milliSiemens; ppt, parts per thousand; TAN, total ammonia nitrogen.

Each diet were randomly selected each week to be weighed as a group. The daily ration was adjusted weekly from the average group wet weight calculated for each diet. Rations were measured with a powder measure (Lee Precision, Inc.) calibrated to dispense the ration for each diet. To minimize cross-contamination, each dietary lipid amount had its own powder measure.

Experiment termination.

At the termination of the feeding trial, fish were 4.5 mo of age. Sex and terminal measures of body mass (BM) and total body length (TBL; measured from tip of the snout to tip of the top of the caudal fin) were determined individually for all fish in the study as previously described (36). After recording this information, fish were randomized to evaluation of body composition, reproductive success, or additional outcomes not discussed in the current paper. Zebrafish randomized to reproductive success were returned to the re-circulating system. Fish assigned to all other outcomes were killed by rapid submersion in ice water for a minimum of 10 min (after which time cessation of all opercular movement was observed) and then stored at −20 °C until analysis.

Body-composition assessment.

Twelve males and 12 females from each diet (3 males and 3 females per cohort) were analyzed for body composition (assessed gravimetrically as total body lipid and TG mass). Female zebrafish were ovariolectomized prior to analysis. After removal from −20 °C storage, total body lipid was extracted from whole-fish samples with chloroform and methanol (37) using a protocol modified for zebrafish that has been described elsewhere (38). TG mass was determined from each total lipid sample by solid-phase extraction with chloroform and methanol (39).

Evaluation of reproductive performance.

From each diet, 40 females (n = 10/tank, 1 tank/cohort) were reserved for evaluation of reproductive success over a 4-wk breeding period. During the breeding period, females were maintained under the same feeding regime and husbandry conditions as described for the feeding trial. As we were primarily interested in the effects of dietary lipid composition on egg production and embryo quality, experimental males were not used in our evaluation of reproductive performance. Instead, randomly selected females from each diet were paired with Artemia-fed broodstock males (AB strain and 4–6 mo of age) from the Aquatic Animal Resource Core at UAB. Each breeding pair represented 1 breeding event, with n = 32 breeding events evaluated per diet. Protocols for breeding and embryo assessment have been described elsewhere (36). Females evaluated for reproduction were killed at the end of the breeding period as previously described.

Spawning success rate was defined as the proportion of successful breeding events (eggs released) to total breeding events. Total egg production represented the number of eggs produced from each individual clutch (breeding event). Embryo viability was determined from successful breeding events as the proportion of viable embryos to total eggs. Embryos exhibiting a stage of development consistent with the pharyngula period at 24–30 h postfertilization (hpf) were considered viable (40).

Statistical analysis

Sample sizes for growth and body composition parameters are given in Table 2. Lengths for 16 male and 27 female zebrafish were unable to be measured from photographs. Measures of total lipid mass (TLM) were missing from 9 samples (6 males and 3 females) due to loss in storage, while TG mass was unable to be measured in 26 samples (10 males and 16 females) due to malfunction of sample-processing equipment.

All data analyses were performed with R Statistical Software (R Core Team, 2016, version 3.4.2) were 2-tailed, with P < 0.05 considered statistically significant. Figures were produced with the “ggplot2,” “ggpubr,” and “metR” packages. All analyses evaluated main and interactive effects of total dietary lipid and ratio of n–6 to n–3 fatty acids. Diet-by-sex interactions were analyzed when applicable, and when significant, analyses for these outcomes were stratified by sex. All models controlled for cohort as either a random effect (growth outcomes) or fixed effect (body composition and reproduction outcomes). Unless otherwise indicated, outcomes calculated as percentages were log-transformed prior to analysis. For categorical variables (both dietary lipid components and week), differences were analyzed relative to the reference group (intercept).

Differences in BM and length were evaluated with linear mixed-effects regression analysis [“lme4” and “lmerTest” packages in R (44, 45)]. Analyses of both outcomes controlled for tank as a random effect. Body-composition differences were assessed with additive effects regression analysis [“gamlss” package in R (46)], with the most parsimonious model selected for each outcome.

Differences in total egg production were evaluated with a zero-inflated negative binomial regression model (46, 47), while embryo viability was assessed with a zero-inflated B regression analysis (46). The zero-inflated B regression model used the parameters μ (location)
Table 2: Sample sizes of male and female zebrafish evaluated for outcomes in growth and body composition, by dietary lipid component and individual diets

| Dietary Component | Wet body mass, n (M | F) | Total body length, n (M | F) | Total lipid mass, n (M | F) | TG mass, n (M | F) |
|-------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|
| TDL (g/kg)        |                     |                     |                     |                     |                     |                     |                     |
| 80                | 239                 | 370                 | 234                 | 353                 | 35                  | 36                  | 32                  | 28                  |
| 110               | 238                 | 369                 | 232                 | 363                 | 32                  | 33                  | 32                  | 33                  |
| 140               | 222                 | 356                 | 217                 | 352                 | 35                  | 36                  | 28                  | 28                  |
| RFA               |                     |                     |                     |                     |                     |                     |                     |                     |
| 1.4:1             | 206                 | 396                 | 197                 | 385                 | 34                  | 35                  | 27                  | 28                  |
| 4:1               | 261                 | 345                 | 257                 | 341                 | 34                  | 36                  | 34                  | 36                  |
| 9.5:1             | 232                 | 354                 | 229                 | 342                 | 35                  | 34                  | 31                  | 30                  |
| Individual diet   |                     |                     |                     |                     |                     |                     |                     |                     |
| 80 g/kg TDL       |                     |                     |                     |                     |                     |                     |                     |                     |
| 1.4:1             | 69                  | 126                 | 66                  | 119                 | 12                  | 12                  | 9                   | 9                   |
| 5:1               | 92                  | 117                 | 91                  | 116                 | 11                  | 12                  | 11                  | 12                  |
| 9.5:1             | 78                  | 127                 | 77                  | 118                 | 12                  | 12                  | 12                  | 12                  |
| 110 g/kg TDL      |                     |                     |                     |                     |                     |                     |                     |                     |
| 1.4:1             | 70                  | 138                 | 66                  | 137                 | 10                  | 11                  | 10                  | 11                  |
| 5:1               | 89                  | 120                 | 87                  | 118                 | 12                  | 12                  | 12                  | 12                  |
| 9.5:1             | 79                  | 111                 | 79                  | 108                 | 10                  | 10                  | 10                  | 10                  |
| 140 g/kg TDL      |                     |                     |                     |                     |                     |                     |                     |                     |
| 1.4:1             | 67                  | 132                 | 65                  | 129                 | 12                  | 12                  | 8                   | 8                   |
| 5:1               | 80                  | 108                 | 79                  | 107                 | 11                  | 12                  | 11                  | 12                  |
| 9.5:1             | 75                  | 116                 | 73                  | 116                 | 12                  | 12                  | 9                   | 8                   |

1 RFA, dietary ratio of n-6 to n-3 fatty acids; TDL, total dietary lipid; TG, triglyceride.

**Results**

Survivorship surpassed 95% for all diets (data not shown), which was comparable to what has been observed in other zebrafish nutrition studies (14, 36). All diets promoted growth and weight gain over the course of the study, with no apparent limitations in palatability observed.

Both wet BM (Figure 1 and Supplemental Table 4) and TBL were significantly higher in female zebrafish (BM: males = 331 ± 3.94 mg and females = 509 ± 5.65 mg; TBL: males = 32.6 ± 0.120 mm and females = 34.8 ± 0.123 mm; P < 0.001 for both outcomes). Sex-by-diet interactions were observed for both outcomes (BM: P-interaction = 0.015; TBL, P-interaction = 0.003). In males, BM was negatively associated with total dietary lipid and ratio of n-6 to n-3 fatty acids; additionally, these dietary lipid components significantly interacted in their effects on BM. Female BM was not significantly associated with either dietary lipid component, although a trend for a negative association with total dietary lipid was observed (P-trend = 0.060). In both sexes, total dietary lipid was negatively associated with TBL and a significant interaction with the ratio of n-6 to n-3 fatty acids was observed (males, P-interaction = 0.003; females, P-interaction = 0.020) (Supplemental Table 4).

**Body composition**

Diet and sex significantly interacted in their effects on TLM (P-interaction = 0.029). TLM was negatively associated with the ratio of n-6 to n-3 fatty acids in male zebrafish and positively associated with total dietary lipid in female zebrafish (Figure 1 and Supplemental Table 5). A significant total dietary lipid by ratio of n-6 to n-3 fatty acids was also observed in females. Similar to TLM, TG mass in males was inversely associated with the ratio of n-6 to n-3 fatty acids (Table 3 and Supplemental Table 6). In contrast to TLM, TG mass in females was not significantly associated with either dietary lipid component, and no evidence of a significant total dietary lipid by ratio of n-6 to n-3 fatty acid interaction was observed in either sex (males, P-interaction = 0.15; females, P-interaction = 0.06).

**Reproduction**

Both total dietary lipid and ratio of n-6 to n-3 fatty acids significantly predicted spawning probability (Table 4). In contrast, egg production was not significantly associated with either dietary lipid component, while embryo viability was only significantly associated with the ratio of n-6 to n-3 fatty acids (Figure 2 and Supplemental Table 7). Females fed diets with an n-6 to n-3 ratio of 5:1 produced the highest proportion of viable embryos. An interaction between the 5:1 ratio of n-6:n-3 ratio and 80 g/kg of total dietary lipid was also observed, as females fed this diet produced the highest proportion of viable embryos (Supplemental Table 8).

It was also noted that breeding trial week was significantly associated with egg production and spawning success. Compared with week 1, egg production was higher in weeks 3 and 4 (P < 0.001; data not shown), while spawning success increased in weeks 2, 3, and 4 (Table 4).

**Discussion**

Our results demonstrate that both total dietary lipid and ratio of n-6 to n-3 fatty acids significantly impact growth, body composition, and reproductive success. We report interactions of these 2 dietary compo-
Both increasing total dietary lipid and ratio of n–6 to n–3 fatty acids were independently associated with reduced terminal BM in male, but not female, zebrafish. In males, the negative association of dietary n–6 fatty acids with BM conflicts with results from previous studies, which
found either positive or no associations between these variables (14, 48, 49). One potential explanation is that only 1 amount of total dietary lipid was utilized for the diets in previous studies, while results from the current study reflect a different effect for the ratio of n–6 to n–3 fatty acids when evaluated across multiple dietary lipid amounts. The negative association between BM and total dietary lipid in male zebrafish also conflicted with previous studies in adult zebrafish (26, 27, 29, 50). However, a similar negative association between growth performance and amount of total dietary lipid was also observed in juvenile turbot, halibut, and Sengalese sole, indicating that life stage may significantly influence the response to dietary lipid (51–53).

Variations in nutrient requirements, utilization, and partitioning among life stages may influence these differential responses. It has been suggested that diets composed of 32% protein are sufficient to meet the growth requirements of older zebrafish, but for juveniles, the requirement is higher (40%, or 14 mg·g·BM⁻¹·d⁻¹ for maximal growth) (54). While the diets in our study were isonitrogenous, the protein-to-energy balance differed, which can significantly impact efficiency of protein retention. Previous research has suggested that there is an optimal ratio for zebrafish (51, 54, 55). Fish consuming the lowest amount of dietary lipid had the highest growth performance, suggesting that these diets promoted the best feed conversion and protein efficiency ratios. Alternatively, an excess of energy intake and a diet with an improper protein-to-energy balance may decrease protein gain and retention with deleterious effects on growth performance, which would provide an explanation for declining growth as dietary lipid amounts increased in the present study (54). Juvenile zebrafish consuming diets with a higher lipid content may not have been able to consume enough protein to meet requirements, and consequently were smaller than those fed the low-lipid-content diets. Results from our study and previous studies indicate that both life stage and nutrient ratios should be carefully considered when designing translational nutrition studies in zebrafish.

Sexual dimorphisms in lipid metabolism and adipose tissue deposition have been observed in both mammals and fish, and are believed to be primarily regulated by sex hormones (1, 23, 56). Estrogens promote the allocation of body fat to subcutaneous depots in females, while testosterone shifts body fat to abdominal and visceral depots in males. Estrogens have also been observed to protect against weight gain and increases in adiposity by increasing energy expenditure rates in humans and mice (4, 50, 56–58). These differences in body fat deposition and metabolism can potentially contribute to variations between men and women in the processing and allocation of dietary lipid (59, 60).

In our study, the ratio of n–6 to n–3 fatty acids was only associated with total lipid mass in male zebrafish. Consistent with our results, sex-specific responses to dietary fatty acid composition have also been described in humans. Dietary fatty acid content has been observed to have a larger effect on the postprandial lipemia response in men compared with women (57, 59). As visceral fat is associated with an increased risk of cardiovascular disease and metabolic dysfunction in humans and zebrafish (26, 61, 62), the higher sensitivity to metabolic disturbances in response to alterations in dietary fatty acid content could be influenced by the larger proportion of visceral fat in males. While dietary fatty acid content was not observed to significantly affect body composition in these short-term studies, another study reported a positive association between visceral adiposity and the postprandial TG response in both men and women (60). Therefore, it could be speculated that, in longer-term studies, acute variations in the postprandial TG re-

### TABLE 3
Terminal triglyceride mass content of male and female zebrafish by dietary lipid component

| Total dietary lipid (g/kg) | Percentage of dry body mass | Male   | Female   |
|---------------------------|----------------------------|--------|----------|
|                           |                            |        |          |
| 80 (reference)            | 100.0 ± 0.16               | 100.0  | 100.0    |
| 110                       | 98.0 ± 0.20                | 98.0   | 98.0     |
| 140                       | 96.0 ± 0.24                | 96.0   | 96.0     |
| Dietary ratio of n–6 to n–3 fatty acids |                     |        |          |
| 1.4:1 (reference)         | 100.0 ± 0.16               | 100.0  | 100.0    |
| 5:1                       | 98.0 ± 0.20                | 98.0   | 98.0     |
| 9:5:1                     | 96.0 ± 0.24                | 96.0   | 96.0     |

1 Values are percentage mean ± SEM and represent measures obtained at termination of the 16-wk feeding trial. Differences were evaluated with additive effects regression and analyzed separately in males and females. **Different from the reference group (P < 0.01) within each dietary lipid component. NS, P > 0.05.

### TABLE 4
Spawning success of female zebrafish by dietary lipid component and week

| Total dietary lipid (g/kg) | Successful/total breeding events | Proportion successful | Estimate(SE) | P  |
|---------------------------|----------------------------------|-----------------------|--------------|----|
|                           |                                  |                       |              |    |
| 80 (reference)            | 72/96                            | 0.75                  | – 0.22 (0.33) | 0.51|
| 110                       | 67/96                            | 0.70                  | – 0.70 (0.32) | 0.030|
| 140                       | 57/96                            | 0.59                  | – 0.69 (0.33) | 0.038|
| Dietary ratio of n–6 to n–3 fatty acids |                     |                       |              |    |
| 1.4:1 (reference)         | 75/96                            | 0.78                  | – 0.74 (0.33) | 0.026|
| 5:1                       | 61/96                            | 0.64                  | – 0.69 (0.33) | 0.038|
| 9:5:1                     | 60/96                            | 0.63                  | – 0.74 (0.33) | 0.026|
| Week                      |                                  |                       |              |    |
| 1 (reference)             | 39/72                            | 0.54                  |              |    |
| 2                         | 51/72                            | 0.71                  | 0.82 (0.36)  | 0.048|
| 3                         | 50/72                            | 0.69                  | 0.68 (0.36)  | 0.06|
| 4                         | 53/72                            | 0.74                  | 0.97 (0.37)  | 0.009|

1 Assessed over a 4-wk breeding period following termination of the 16-wk feeding trial. Successful events = female released eggs; n = 32 total breeding events per diet.

2 Estimates are from the logistic component of zero-inflated negative binomial regression analysis and represent probability of a successful spawn relative to the reference group within each predator variable. Significant P values (P < 0.05) represent differences from the reference group.
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response to dietary manipulation could translate to changes in adiposity over time (60).

Conversely, a significant association between total dietary lipid and TLM was only observed in female zebrafish in our study. In mice, previous studies have demonstrated that males consuming an HFD are more susceptible to metabolic dysregulation, inflammation, and glucose intolerance (63–65). It could be speculated that this increased sensitivity in males could result in a stronger drive to adjust feed intake relative to energy density of the diet, and explain why total dietary lipid was positively associated with TLM only in females (56).

In vertebrates, TGs are predominantly associated with fat mass storage, with main storage sites including visceral, intramuscular, and subcutaneous depots (28). Analogous to total body lipid, body TG mass in males was significantly associated with the ratio of n–6 to n–3 fatty acids. In contrast, body TG mass in females was not influenced by either dietary lipid component. We are unsure as to why no significant effects of diet were observed for TG mass in females, indicating that additional investigations are needed to further explore these outcomes. It is also unclear whether the amounts of stored TG mass observed in our study represent positive or negative effects on health. To answer this question, healthy levels of adiposity will need to be defined for male and female zebrafish by evaluating additional markers for health and fitness.

Reproductive performance is influenced by both dietary lipid quantity and quality in fish and mammals (17, 66–69). In our study, the total amount of dietary lipid was significantly associated with spawning rate, but not total egg production or embryo viability. Spawning rate was observed to be lower in females consuming higher amounts of dietary lipid. This observation conflicts with studies in channel catfish and the snakehead murrel, where spawning rate and dietary lipid amount were positively associated (70, 71). However, the diets of catfish and murrels differ from zebrafish, resulting in diverse requirements for total dietary lipid intake. Zebrafish are a predominantly low-trophic-level species with a lower requirement, and consequently tolerance, for total dietary lipid intake (72). This lower tolerance may translate to a reduced ability to effectively utilize large amounts of dietary lipid for energy, resulting in increased ectopic fat storage (71–73).

Previous research in mice has demonstrated that ectopic fat deposition in ovaries and other tissues resulting from diet-induced obesity initiates a cascade of lipid-induced programmed cell death known as lipotoxicity (74, 75). Consequently, female mice fed HFDs exhibit impaired oocyte release and fecundity. Analogous to mammals, zebrafish express markers associated with lipotoxicity in response to HFDs; therefore, higher amounts of dietary lipid could negatively impact spawning success in female zebrafish through a similar mechanism (26, 76). The total dietary lipid amounts selected for this study were presumed to be within a healthy range, which may provide an explanation for why neither total egg production nor embryo viability were affected. If diets with total lipid amounts outside this range were included in the study, larger differences in reproductive success among diets may have been observed (72).

It has been well established that mechanisms affecting egg release are influenced by dietary n–6 and n–3 fatty acid intake (67, 77, 78). These fatty acids regulate and serve as precursors to a group of physiologically active lipid compounds known as eicosanoids. ARA is a precursor for proinflammatory series-2 prostaglandins [prostaglandin E2 (PGE_{2}) and prostaglandin F_{2alpha} (PGF_{2alpha})], while EPA (20:5n–3) and DHA (22:6n–3) are precursors for the less inflammatory series-3 prostaglandins (PGE_{3}) (77, 79). Ovaries have a high capacity to generate these prostaglandins, which affect gonadal steroidogenesis and ovulation in both mammals and fish (77, 80, 81). The negative association between spawning rate and ratio of n–6 to n–3 fatty acids observed in our study is supported by previous studies that evaluated ovulation and egg release in humans and animal models (6, 66, 67, 73, 78, 79). While production of PGF_{2alpha} is necessary for ovulation and spawning behavior in mammals and fish, administration of PGE_{3} has been shown to inhibit ovulation (78, 82). Increasing dietary intake of n–3 fatty acids may enhance ova release by reducing production of PGE_{2}, potentially through reduced ARA availability and elevated PGE_{3} synthesis (79).

Similar to total dietary lipid, the ratio of n–6 to n–3 fatty acids was not associated with total egg production. However, the significant association between embryo viability and dietary ratio of n–6 to n–3 fatty acids observed in our study reflects a hormetic dose–response relation. While the proportions of viable embryos produced by females fed diets with 1.4:1 and 9.5:1 ratios were similar (76% and 75%, respectively), embryo viability was significantly higher for the 5:1 ratio (87%). Hormetic
relations have been described in variable intakes of other macronutrients or micronutrients and are believed to occur through a phenomenon known as "Bertrand's rule" (83–85). With Bertrand's rule, an increase in the intake of a specific nutrient is associated with increasing health benefits until reaching an optimal intake; beyond this optimal intake, further increases in intake may lead to adverse health consequences (83, 84). In zebrafish, the 5:1 ratio may represent an optimal intake ratio of n–6 to n–3 fatty acids for embryo viability, whereas the 1.4:1 and 9.5:1 ratios may represent imbalances in the ratio of n–6 to n–3 fatty acids.

The dietary ratio of n–6 to n–3 fatty acids may have also influenced embryo viability through spawning interactions. In teleosts, prostaglandins also act as pheromones to induce male spawning behavior and fertilization (86). The effects of dietary n–6 and n–3 fatty acids on prostaglandin production and release in females could influence signaling mechanisms for sperm release, ultimately affecting whether eggs are fertilized. In addition to their effects on female fish, another study found that ARA and EPA also modulate steroidogenesis in goldfish testis (87). However, caution should be exercised in extrapolating results from these previous studies to effects of dietary n–6 and n–3 fatty acids on production and release of prostaglandins and steroidogenesis in males or females, as influences of diet were not considered in either of these previous studies. Rather, findings from these studies merit further investigation.

A second factor that may have impacted embryo viability in our study is the effect of dietary long-chain (LC) PUFAs on oocyte development and quality (69). In both mammals and fish, the fatty acid composition of the ovaries is significantly influenced by maternal dietary LC-PUFA intake and is known to influence egg quality (17, 88). In particular, both n–6 and n–3 fatty acids are required for normal oocyte development, and an optimal ratio would improve both egg morphology and hatching rates (48, 77). In marine species, higher egg quality was positively associated with increased ARA and DHA/EPA content (89). However, optimal intakes of dietary n–6 fatty acids and ARA:EPA ratios are likely to be species dependent and influenced by the geography and ecosystem of the species’ food sources (90). This may explain the discrepancy between our results and those from previous studies in marine species. Whether the higher proportion of viable embryos observed from females fed the 5:1 ratio in our study is attributed to spawning behavior, egg quality, or a combination of both is a topic that should be explored in future studies.

Strengths of our study reside in the use of chemically defined diets with purified ingredients, the administration of daily rations during the feeding trial, and the evaluation of sex-specific responses to dietary lipid composition on obesity-related phenotypes. The duration of the feeding trial allowed us to examine the long-term effects of dietary lipid on our outcomes of interest. However, our study also had some intrinsic limitations. We had a female-biased sex ratio of zebrafish in our study sample, which may lead to aggressive interactions and the production of dominant individuals that control access to food resources (91). These behaviors could potentially influence our outcomes evaluated, and in future studies may be attenuated with an even distribution of males and females in each tank (91, 92). The time period of our study included the juvenile life stage of the zebrafish. Inclusion of this life stage may have potentially confounded effects of the diets on weight gain, as nutrient requirements and energy allocation differ from the adult life stage (72, 93). Thus, adult zebrafish may be a better choice for translational applications relating to diet-induced obesity in future studies. Given that vitamin E content is significantly associated with gonad development, fertility, and larval survival rate in fish (77), an additional limitation is that we did not control for variations in vitamin E content between the safflower and menhaden oils. Finally, a major challenge in all zebrafish nutritional studies is accurate measurement of feed intake due to the potential leaching of nutrients in the water (94). While we attempted to address this issue with administration of a daily ration, the development of direct methods for measuring feed intake in zebrafish should continue to be explored (94).

In summary, while requirements for total intake of dietary lipid and ratio of n–6 to n–3 fatty acids in both human and animal diets need to be defined, our results demonstrate that the balance of macronutrients may be as important as their individual intake. Dietary lipid quality and quantity exhibited independent and interactive effects on weight gain and reproductive success, suggesting that maximum benefits from their intake may be reached only when they are in the appropriate proportions (14). Optimal intakes of dietary lipid may also vary by sex. Our observations for body composition suggest that processes mediating the partitioning and utilization of dietary lipid are sexually dimorphic. Identification of sex-specific physiologic responses to dietary manipulation could lead to improved treatment and prevention strategies for obesity. Findings from this study and other studies continue to validate the zebrafish as a high-throughput model to identify underlying mechanisms that contribute to the development of diet-induced obesity, which will ultimately contribute to the development of sex-specific therapies (24).

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