STANDARDIZING AND OPTIMIZING REARING METHODS OF ANIMAL MODELS: INFLUENCE OF DIET ON GROWTH AND REPRODUCTION IN *XIPHOPHORUS MACULATUS* AND *ORYZIAS LATIPES*

by

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ABSTRACT

In research, reproducibility is the ability to duplicate the results of a previous study using the same materials and methods as the original researchers. Fish models of human diseases have become more popular in the last thirty years. However, husbandry practices affect the reproducibility of a study. In most laboratories, fish are reared on a custom blend of live feed and other ingredients or a commercial diet that discloses minimal nutritional information. Further, researchers rarely publish the nutritional content of feeds with findings. Overall, this lack of standardization and reporting may confound research outcomes and lead to unreliable inter-laboratory reproducibility. Accordingly, the objective of our study was to determine how diet affects research outcomes. To achieve this, two biomedical fish models, *Xiphophorus maculatus* (platy fish) and *Oryzias latipes* (medaka), were used to evaluate dietary effects on growth and reproductive performance. Platy fish (n =120) and medaka (n =300) were reared from birth to one month of life on Ziegler Aquatox flakes and live *Artemia nauplii,* then randomly divided into groups fed one of three diets for six months: a control diet (CON), consisting of Ziegler Aquatox flakes, live *Artemia nauplii,* and beef liver paste (liver paste excluded for Medaka); a commercially available zebrafish diet (GEM); or a laboratory defined reference diet (WAT). Fish were fed 3% body weight/day and grouped at similar stocking densities. At baseline and in monthly increments thereafter, individual fish weight, total length, and width were assessed. Beginning at 3 months of age, broods were raised from platy fish as they were born and eggs were collected from medaka bi-weekly. Data were analyzed in

RStudio using a mixed model ANOVA with diet as a fixed factor and tanks nested as random factors within diet. If data were normally distributed ($P > 0.01$ on Shapiro-Wilks test), a parametric ANOVA test was performed and post hoc analyses and pair-wise analyses were conducted using *t*-tests. Non-normally distributed data ($P \leq 0.01$ on Shapiro-wilks test) were evaluated using the Kruskal Wallis rank sum test. In case of significant results, *post hoc* testing using the Nemenyi tests for multiple comparisons of rank sums was performed. Diet affected growth measures for both platy fish and medaka. We observed significant differences in percent change of length, weight, and width from baseline through 6-months of age for both species of fish. However, there were not many differences between dietary groups in body condition factor at baseline, 3-months, or 6-months for either species. Significant differences were also observed in reproductive measures for both fish species. In platy fish, only one dietary treatment (CON) produced fry. In medaka, there were dietary group differences in the number of eggs collected and percent survival of hatched eggs. These data indicate that diet affects growth and reproductive outcomes even when feeding protocols are otherwise identical, hindering reproducibility of research if diet is not controlled or reported. Collectively, our data emphasize the need for research laboratories to develop standardized feeding practices, report dietary data, and define reference diets based on fish species.

I. INTRODUCTION & REVIEW OF LITERATURE

INTRODUCTION

Animals are often used in research to model human diseases. Beyond the rigor and reproducibility of these experiments, there are additional advantages to using animal models in research, including standardized care controls and availability of inbred strains to reduce the confounds of genetic discrepancies (Osier, et al., 2016). When rearing and maintaining laboratory animals, environmental factors should be considered including, but not limited to, housing conditions, temperature, humidity, light and dark cycle timing, and diet. These factors are assessed for ethical integrity by an Institutional Animal Care and Use Committee (IACUC) prior to execution of an experiment; however, it the researcher's responsibility to ensure their experiments are scientifically sound and measure the intended outcome. Unless directly studied, diet is an environmental factor that is often overlooked as a possible confounding variable to research outcomes. Dietary composition and nutritional value are also often not reported in the methods sections of publications (Pellizzon & Ricci, 2020).

Dietary variations can result in phenotypic and genotypic differences in laboratory animals (Kozul et al., 2008). Adequate nutrition and proper feeding practices are necessary for embryo development, growth, reproduction, and health of any organism. Additionally, maturation and reproduction are often the most energetically taxing periods of life due to energetic resources being diverted from procurement of food and into tasks necessary for growing and breeding. Thus, these life stages have differing nutritional

requirements to maintain homeostasis and, if those needs are not met, animals may perform poorly.

Fish are commonly used laboratory animals for studies in molecular genetics, behavioral genetics, carcinogenicity, and toxicology (Kazianis & Walter, 2002; Loosli et al., 2000; Godfrey et al., 2019). The debut of *Danio rerio,* also known as the Zebrafish, in the mid-twentieth century paved the way for fish models in research (Westerfield, 2000; Teame et al., 2019; Winata et al., 2020). At the molecular level, there are few differences between humans and fish; for example, one of the most frequently mutated cancer genes, HRAS, shares >95% identity with the corresponding gene in the fish species medaka (Schartl, 2015).

Xiphophorus maculatus, also known as the southern platy fish, is a freshwater fish species originating from the Rio Jamapa drainage, Veracruz, Mexico. *Xiphophorus maculatus* have been cultivated since 1971 (Walter et al., 2006), hybridize well in captivity, and their interspecies hybrids have a high degree of heterozygosity that make them valuable models for molecular genetic studies (Walter, et al., 2006). Research using platy fish has increased understanding of the genetic mechanisms underlying tumorigenesis, including spontaneous and induced neoplasia and several types of malignant melanomas (Walter et al., 2006). Additionally, these fish are used in many other areas of research such as behavioral genetics, studies in mechanisms of sexual selection, and molecular events leading to speciation (Walter et al., 2006).

Oryzias latipes, also known as the Japanese medaka, is a freshwater fish species indigenous to Japan, Korea, Vietnam, and China (Leaf et al., 2011). Medaka are used in many biological fields of research such as developmental biology, genetics, and

embryology due to their high fecundity rates, population growth rates, and ease of husbandry (Leaf et al., 2011). They tolerate a wide range of temperatures and can, thus, be cultivated with outdoor or indoor methods. Medaka are advantageous for research due to their high rates of transgenesis, suitability for bioimaging analyses due to the transparency of embryos and larvae, and for their compatibility with high throughput chemical screens (Schartl et al., 2015). Additionally, medaka melanoma models mimic the appearance of melanocytes in the human epidermis making medaka an important species for malignant melanoma research (Schartl et al., 2015).

ANIMAL MODELS

Laboratory animals are used in research to describe a biological phenomenon that a specific species has in common with a target species (Hau & Schapiro, 2010), most often, humans. An animal in which normative biology or behavior can be studied, or in which a pathological process can be investigated, and in which the phenomenon resembles the same phenomenon in humans or another target species is an animal model (Hau & Schapiro, 2010). Animal models have been key in developing treatments for asthma, cancer, HIV/AIDS, and many more (Graffi et al., 2002; Giles et al., 2015). Additionally, they play a crucial role in developing antibiotics, vaccinations, and organ transplantation techniques (Cavalcante et al., 2019).

Although animal models have contributed a large amount to our understanding of various human processes, diseases, and treatments, many investigators have questioned their reliability and assert that improvements need to be made in preclinical animal

research to produce the best predictions for reliability in human studies (Martic-Kehl et al., 2012). There are two types of established reliability in research – extrapolation and experimental.

Under defined test conditions, a model is *extrapolation* reliable if the results produced by the model mirror the results produced by an identical test on a human subject (Rohra & Qazi, 2008). However, human studies are difficult to conduct due to lack of standardized care controls and ethical concerns. In contrast, under defined test conditions, an animal model is *experimentally* reliable if the results can be reproduced by subsequent tests and by different researchers following an identical protocol (Rohra & Qazi, 2008). If environmental factors such as diet, housing conditions, and handling practices are standardized, then that model has *experimental* reliability, which paves an easier path to *extrapolation* reliability. Traditional animal models (i.e., mice) have strict standardized husbandry practices to make them reliable models (Mandillo et al., 2008; National Research Council, 2011).

STANDARDIZATION OF DIETS

In research, reproducibility is the ability to duplicate the results of a previous study using the same materials and methods as the original researcher. Reproducibility of laboratory experimental results has been improved through microbiological and genetic definitions in animals, but variation is still introduced or exacerbated by other controllable environmental factors, one of the most common being diet. In 1974, the Medical Research Council of Laboratory Animals Centre created the Laboratory Animals

Diet Committee (LADC). Since 1977, when the first Dietary Standards for Laboratory Animals Report was released (Clarke et al., 1977), it has been updated once in 1995. Additionally, the National Research Council created the "Guide for Care and Use of Laboratory Animals" which was last updated in 2011. However, this guide does not specify in depth how to feed laboratory animal fish except that it should be species dependant (National Research Council, 2011).

Fish models of human diseases have grown in popularity over the last thirty years. However, fish husbandry practices have not been standardized and can widely affect study outcomes and inter-laboratory reproducibility. Specifically, researchers use a wide variety of commercial and/or "home-made" diets that are specific to their laboratory. These diets vary substantially in composition, nutritional value, and inclusion of unidentified constituents (e.g., preservatives or toxins). Further, dietary characteristics are often not reported by researchers, limiting the ability of other laboratories to achieve reproducibility and, ultimately, reliability.

Natural-ingredient diets are those formulated with ingredients such as whole grain, mill by-products, high protein meals, mined or processed minerals, or other livestock feed ingredients (National Research Council, 1995). These diets are the most commonly used commercial diets for laboratory animals because they are relatively inexpensive and highly palatable (National Research Council, 1995). However, variations in the composition of individual ingredients change nutrient concentrations of the overall feed, resulting in no two batches being identical and limiting reproducibility over time. The potential for contamination with natural-ingredients diets is also high due to pesticide residues and heavy metals (National Research Council, 1995).

An alternative approach to natural-ingredient diets is fixed-formula diets (National Research Council, 1995), diets in which the kinds and amounts of ingredients do not vary from batch to batch. Fixed formula diets are often referred to as open-formula diets as the formula is openly declared (National Research Council, 1995). Fixed-formula diets may contain multiple sources of protein, fat, and carbohydrates, thereby reducing the importance of variation in the composition of any particular ingredient from batch to batch. Additionally, these diets increase the probability that trace minerals of potential nutritional importance are included (National Research Council, 1995).

Fixed-formula diets have been altered even further to create purified or chemically defined diets. Purified diets are formulated with a more refined and restricted set of ingredients than fixed-formula diets and have a low chance for chemical contamination. Only relatively pure and invariable ingredients are used in these formulations. In contrast, chemically defined diets represent the highest degree of control over nutrient concentrations and can be sterilized for use in germ-free and low antigen studies (National Research Council, 1995).

A laboratory animal's nutritional status affects its ability to reach its genetic potential for growth, reproduction, and longevity (National Research Council, 1995). The welfare of laboratory animals as well as unbiased experimental results depend on a nutritionally balanced, standardized diet. Nutrient requirements have thus far been established for rats, mice, guinea pigs, hamsters, gerbils, voles, rabbits, and primates (National Research Council, 1995), however, have not been established in laboratory fish. Given the breadth of research performed in fish models, this poses a systemic challenge for reproducibility of experimental results.

Zebrafish (*Danio rerio*) are small freshwater teleosts that emerged during the 1980s as an experimental model for developmental biology studies (Link & Megason, 2008). Recent studies have demonstrated that zebrafish reared on different diets vary greatly in length, weight, age at sexual maturation, fecundity, and mortality (Siccardi et al., 2009; Gonzales 2012; Fang et al., 2013; Fowler et al., 2019). However, impacts of diet on fish health and behavior for research outcomes have not been well described. Additionally, there is a lack of consensus among different aquatic facilities and commercial vendors regarding nutritional requirements for fish and if these requirements are specie specific. Cumulatively, the lack of diet standardization and reporting may lead to unreliable research outcomes and an inability for laboratories to achieve experimental reproducibility.

FISH AS RESEARCH MODELS

There are many characteristics that contribute to fish's importance as biomedical research models. As vertebrates that live in water, they have remarkable morphological and physiological adaptations that provide a wealth of information regarding sensory transduction mechanisms, biomechanics, and physiology (Fabacher & Little, 2000). Respiration is typically facilitated by gills and fish have two chambered hearts that circulate venous blood. They also have unique sensory abilities not present in rodents such as response to light and production and detection of electrical impulses (Fabacher & Little, 2000). Additionally, some fish are hermaphrodites while others exhibit sex reversal capabilities (Sakae et al., 2020).

Another physical feature of fish that makes them excellent research models is that they are poikilotherms. Thus, they regulate their body temperature through temperature of the surrounding water, allowing researchers to evaluate certain biochemical and metabolic pathways at different rates through manipulation of water temperature (Fabacher & Little, 2000). As fish are taxonomically and environmentally diverse compared to mammals, researchers also have a wide range of species to investigate their desired research questions.

Teleosts, such as zebrafish and medaka, are increasingly used as animal models in biological research. Medaka are hardy and can tolerate a wide range of temperatures and breed readily in variable environments (Ishikawa, 2000). Embryos and chorions of medaka are transparent and can be easily phenotyped (Ishikawa, 2000). Medaka eggs hatch seven days after fertilization and fry grow and sexually mature in three months (Ishikawa, 2000).

It has been proposed that the longitudinal fiber systems in all vertebrate brains pass through a common layout defined by conserved genetic and developmental programs (Ishikawa et al., 2004). Medaka brains have similar axonal growth patterns in their brains as mice, even more so than zebrafish, making them superior developmental models with respect to mammals (Ishikawa et al., 2004). Medaka are also suitable for large scale mutagenesis at a rate that is comparable to mice and zebrafish (Loosli et al., 2000). The high number of genetic mutations that can be isolated in medaka provide a useful system for detailed analysis of specific aspects of vertebrate development (Loosli et al., 2000).

Cancer and tumor suppressor genes and key signaling pathways, such as those

related to DNA damage and apoptosis, are conserved between fish and humans (Patton et al., 2011). For example, zebrafish and humans share molecular signatures of the progressive stages of liver neoplasia (Patton et al., 2011). Fish tumors share many salient features with cancers derived from analogous tissues in humans discovered via histopathological analysis (Patton et al., 2011).

Fish of the genus *Xiphophorus* are composed of 26 species that are divergent in their external morphology. In *Xiphophorus*, melanoma can be initiated by simple crossings and the signaling pathways governing tumor growth and progression can be delineated (Meierjohann & Schartl, 2006). Melanoma progression models in fish can be viewed as a starting point for identifying novel genes, environmental conditions, and therapeutic compounds that affect this type of cancer (Patton et al., 2011). The genetic system of *Xiphophorus* offers the opportunity to understand tumors on different levels of organization (e.g., molecules and their interactions up to the whole organism; Meierjohann & Schartl, 2006).

FISH PHYSIOLOGY AND DIGESTION

Fish skin is a multifunctional organ that serves not only as protection but also in communication, sensory perception, locomotion, respiration, ion regulation, excretion, and thermoregulation (Elliot, 2000). Specialized sensory structures, such as the lateral line system and chemoreceptor structures (taste buds), are also located in fish skin (Elliot, 2000). Recognizing, acquiring, and initial processing of food is controlled by the mouth, oral cavity, and pharynx (Buddington & Kuz'mina, 2000). The mouth opens into the

buccal cavity and, once food is swallowed, it enters the alimentary canal and proceeds via the esophagus to the stomach and then intestines (Buddington & Kuz'mina, 2000). The passage of ingesta through the medaka digestive tract is portrayed in Figure 1.

The esophagus is the first region of the alimentary canal and is characterized by longitudinal folds that expand to accommodate food passing into the stomach (Buddington & Kuz'mina, 2000). The stomach has three distinct regions: the cardiac, fundus, and pylorus. The cardiac region is directly connected to the esophagus, is non-secretory (Buddington & Kuz'mina, 2000), and functions mainly as storage. The fundic and pyloric regions are secretory and have thicker, muscular walls that mechanically break down food and mix it with acidic gastric secretions (Buddington & Kuz'mina, 2000). Chyme, the mixture of ingested food and acidic gastric sections, is regulated for passage to the intestines through the pyloric sphincter which ensures enzymatic and absorptive capacities of the intestines are not exceeded (Buddington & Kuz'mina, 2000).

The principal site of digestion in fish is the small intestine, the characteristics of which are highly variable depending on the diversity of feeding habits and functional demands of the fish (Buddington & Kuz'mina, 2000). The most visible difference in the intestines between fish species is the length from the pyloric sphincter to the anus or vent (Buddington & Kuz'mina, 2000). Fish lack a distinct colon and functions of the small intestine (i.e., digestion and absorption) can be detected throughout the entire length of the intestines (Buddington & Kuz'mina, 2000). Through use of gene markers, it has been demonstrated that some segments of the medaka small intestine resemble the functions of the mammalian small intestine while others have characteristics of the large intestine despite it being a single, continuous organ, in contrast with that of most mammals

(Aghaallaei et al., 2016).

Fish have developed different anatomical strategies to increase surface area of the digestive epithelium: lengthening the intestines; developing thick mucosa with extensive folding; or developing diverticula, which is an internal epithelial fold that can present as a spiral valve or as pyloric ceca (Buddington & Kuz'mina, 2000). Although the intestinal structure is variable, associated functions are relatively consistent across species. Chyme enters the intestine and is mixed with aqueous secretions from the intestine and other organs (i.e., liver, gall bladder, and pancreas) to digest and absorb nutrients (Buddington & Kuz'mina, 2000). Many marine herbivorous fishes produce short chain fatty acids (also often referred to as "volatile" fatty acids) in the hindgut, which is the latter section of their small intestine (Mountfort et al., 2002). This indicates gut microbial activity and fermentation suggesting that the hindgut portion of the small intestine of fish is analogous to the cecum of mammals.

The liver, pancreas, and gall bladder are also part of the fish digestive tract and remain attached to the alimentary canal by ducts that carry secretions (Buddington & Kuz'mina, 2000). Most of the hepatic (liver) tissue is dedicated to metabolism. The liver filters blood draining from the intestine before entering systemic circulation and filters toxins, diverting them to the gall bladder (Buddington & Kuz'mina, 2000). Hepatic tissue also produces bile, critical for fat digestion, that drains into and is stored in the gall bladder. The pancreas is both an exocrine and endocrine tissue. Pancreatic exocrine secretions include water, digestive enzymes, and bicarbonate while endocrine secretions include hormones that regulate carbohydrate metabolism (Buddington & Kuz'mina, 2000).

DIETARY EFFECTS ON GROWTH

Specific dietary ingredients, nutrients, and antinutritional factors affect growth and development of different fish species (Table 1). Nutritional control in laboratory fish husbandry is lacking due to undefined nutritional requirements and absence of a standard reference diet (Siccardi et al., 2009).

Metabolizable energy (ME) requirements have not yet been determined for most fish species (Wilson, 2002). Most of the existing reference values have been estimated from dose–response curves, indicating the minimum number of dietary macromolecules that resulted in maximum fish growth (Wilson, 2002). Cessation of growth and weight loss may result from inadequate dietary protein due to the withdrawal of protein from less vital tissues to maintain the functions of more vital tissues (Wilson, 2002). Conversely, if too much dietary protein is supplied, much of it will be deposited as adipose, or fat, in tissues.

There is considerable evidence that many species of fish larvae have limited capacity to biosynthesize linoleic, linolenic, eicosapentaenoic, and docosahexaenoic fatty acids *de novo* (Denny et al., 2004). It is thought that this is also true for the *de novo* biosynthesis of cholesterol and sphingolipids, indicating that dietary sources of these fatty acids and lipids are likely essential for fish growth and survival. However, it is difficult to supplement these nutrients in the diet due to the production of rancidin, an oxidative product that results in high levels of free radicals which are toxic to fish (Roberts, 2002). Rancidin can also result in fatty liver disease and increased adiposity (Roberts, 2002).

Hu et al. (2013) demonstrated that differing protein sources with differing fatty

acid concentrations affected growth of Japanese seabass (*Lateolabrax japonicus*) in terms of specific growth rate and whole-body lipid concentration (Table 1). Six isonitrogenous (44% crude protein) and isoenergetic diets were designed using different animal protein blends (e.g., poultry by-product meal, meat and bone meal, spray-dried blood meal, and hydrolyzed feather meal). The substitution of an animal protein blend for fish meal significantly decreased growth rate and caused fatty liver disease. As many commercial fish feeds have differing ingredients, this can pose a major problem to fish development and inconsistent experimental or general husbandry outcomes.

Another common issue in fish nutrition is the contamination of feeds. There is often heavy metal contamination from storage vessel leaching or use of unusual feed sources (Roberts, 2002). Heavy metal toxicity can significantly inhibit growth, reduce feed conversion efficiency, and cause spinal curvatures (Roberts, 2002). Additionally, mycotoxins are a source of natural contaminants commonly found in fish feed. .The mostcommon mycotoxin are aflatoxins, which are found in oil seeds such as cotton and peanut (Roberts, 2002). Another dietary contaminant with relevance to fish nutrition are soy isoflavones, which are commonly found in aquatic flake foods and mimic the effects of the sex hormone estrogen (Naciff et al., 2004).

Growth differences have been observed in fish due to differing dietary constituents with undefined nutritional requirements (Table 1). Recently, Fowler et al. (2019) fed five common commercial diets and one formulated chemically defined reference diet to exemplify differences in growth outcomes in commonly used feeds in zebrafish laboratories. Significant differences in growth factors (i.e., final weight, body condition index, and fat deposition) were observed. An important factor pointed out by

these researchers is that many aquaculture diets are formulated to produce a fish that grows rapidly, often using metrics related to meat production and not necessarily long-term health (Fowler et al., 2019), which could compromise the outcome of experimental data.

DIETARY EFFECTS ON REPRODUCTION

Reproduction involves dramatic changes in energy acquisition and partitioning by fish (Bureau et al., 2002). Synthesis and temporary storage of new tissues that are formed during the reproductive phases of life occur almost regardless of the level of dietary energy intake (Bureau et al., 2002). In this case, necessary energy will be drawn from other body tissues if the dietary supply is inadequate. In many fish species, the energy required for development of secondary sexual characteristics and reproduction can cost about 60-70% of the body's energy reserves (Bureau et al., 2002).

The average energy content of eggs for teleosts is 23.5 kJ/g dry matter regardless of size of the egg (Kaushik and Medale, 1994). This total amount of energy stored in eggs represents 8-15% of the gross energy of the whole body of the fish (Kaushik and Medale, 1994). Mature ovaries can represent up to 30% of the body mass in certain species of fish (Bureau et al., 2002). This is represented by the gonadosomatic index (GSI) calculated as [gonad weight / total tissue weight] × 100.

Fowler et al. (2019) identified differences in GSI in female zebrafish fed different commercially available fish diets. Diet also affected other reproductive performance measures, such as percentage of successful spawns and embryo viability (Table 2). A

more recent paper by Martin et al. (2021) also noted significant differences in GSI of Senegalese sole (*Solea senegalensis*) when fish were allowed to feed naturally versus fed commercial fish diets. This research also demonstrated distinct differences in male fish via sperm production and motility differences between diets (Martin et al., 2021).

In medaka, researchers feeding increasing levels of polyunsaturated fatty acids observed significantly different fertility rates, embryo survival rates, and differences in sperm motility between diets (Kowalska et al., 2020). Increased amounts of arachidonic acid in the brood stock diet resulted in the highest rates of embryo and larval survival (Kowalska et al., 2020). As demonstrated by this study, differing amounts of dietary constituents can result in significant changes in reproductive performance and ability.

II. OBJECTIVES & METHODS

OBJECTIVES

The objectives of this study were to evaluate how different diets influence growth and reproductive measures in animal models commonly used to research human diseases. Two biomedical fish models – *Xiphophorus maculatus* (platy fish) and *Oryzias latipes* (medaka) – were used to address study objectives. Findings have the potential to provide evidence for the need of an individual species development of a defined diet for future standardization. These findings can enhance laboratory animal husbandry by providing insight into improving fish and other biomedical research species' performance. Additionally, findings have potential to indirectly improve human health through improvement of experimental rigor and reproducibility of biomedical research models.

RESEARCH QUESTIONS

1. What is the relationship between diet and growth (e.g., length, width, weight, and body condition factor (BCF)) from 1 to 6 months of age in platy fish and medaka?
2. What is the relationship between diet and reproduction (e.g., number of live births, numbers of eggs spawned, percent of eggs hatched, and percent survival of hatchlings) between months 3 and 6 of age in platy fish and medaka?

ANIMAL EXPERIMENTS

This study was conducted in accordance with the ethical guidelines for animal research established and approved by the Institutional Animal Care and Use Committee at Texas State University (protocol #7234).

This study was conducted with platy fish (*Xiphophorus maculatus* strain JPWild) and medaka (*Oryzias latipes* strain carbio) obtained from the *Xiphophorus* Genetic Stock Center (XGSC) at Texas State University, San Marcos, TX. Four 10-gallon tanks were prepared for three dietary treatments and two fish species (total of 24 tanks). Tanks were rinsed with deionized water, 1 cup of rocks added, and tanks filled with primed water. One mL of stress-zyme was added to the primed water and allowed to rest for 7 days before addition of fish. Java moss was also added to the tanks to provide eventual fry with shelter. The environmental temperature was maintained at 25°C and fish were maintained on a 13:11 hour light cycle.

Platy fish were reared according to protocols of the XGSC (Walter et al., 2006). Due to the reproductive nature of the platy fish, fry were selected and separated as broods were born. At 1 month of age, platy fish were separated into groups of approximately 40. Each brood was weighed and measured prior to separation into tanks. Groups were then further separated into 4 tanks at similar stocking densities ($n \approx 10$).

Medaka were reared according to Murata & Kinoshita et al. (2019). At 1 month of age, medaka ($n = 312$) were randomly selected from tanks and divided into equal groups (3 groups of $n = 104$). Fish were weighed and measured prior to separation. Groups were then further separated into 4 tanks at similar stocking densities ($n \approx 25$).

TREATMENTS

Fish were fed one of three dietary treatments twice daily for six days per week at 3% body weight (BW)/day (Table 3). On the seventh day of each week, fish were fed 3% BW once daily. Feedings occurred at 0800 h and 1600 h. Diets consisted of the following: a control diet (CON) a dietary regime developed by the *Xiphophorus* Genetic Stock Center consisting of Ziegler Aquatox flake, live *Artemia*, and beef liver paste (BLP, excluded for medaka); the commercial Skretting Gemma Micro150 (juvenile)/300 (adult) (GEM); and a laboratory-defined reference diet developed by Steven Watts at the University of Alabama Birmingham that consists of a high fat (juvenile) then low fat (adult) diet (WAT). Proximate and elemental analysis of all diets was performed by Eurofins Scientific Laboratories, Inc. excluding *Artemia* and BLP (Tables 3 & 4).

Juvenile (birth to 3-months) CON platy fish and medaka were fed 1.5% BW finely ground Aquatox flake and 1.5% BW live artemia split into two feedings. Juvenile GEM platy fish and medaka were fed 3% BW Gemma Micro 150 diet split into two feedings. Juvenile WAT platy fish and medaka were fed 3% BW Watts high fat diet split into two feedings.

At three months of age, all fish were switched to an adult feeding regime. Adult (3-months to 6-months) CON platy fish were fed 0.75% BW Aquatox flake and 0.75% BW live artemia in the morning. At 1600 h, adult platy fish were fed 0.75% BW live artemia and 0.75% BW BLP. Adult CON medaka were fed 1.5% BW Aquatox flake and 1.5% BW live artemia split into two feedings. Adult GEM platy fish and medaka were fed 3% BW Gemma Micro 300 diet split into two feedings. WAT platy fish and medaka were fed

3% BW Watts low fat diet split into two feedings.

ARTEMIA COLLECTION

Artemia were sourced from and nutritional profile was provided by BIO-MARINE® brand Artemia cysts (Table 3). Stage I *Artemia nauplii* were harvested at 0800 and 1400 h daily. *Artemia* cultures were maintained at a water temperature of 25-26°C. Cultures were set up 48 h before harvest in dechlorinated saltwater with a specific gravity range of 1.020-1.024. Before feeding, harvested *Artemia* were strained, rinsed, and resuspended to 100 mg/mL solution with system water.

WATER CHEMISTRY

Tank water quality (alkalinity, CO_2, dissolved O_2, pH, temperature) was tested once monthly and ammonia and nitrite were tested bi-weekly according to the HACH Fish Farming Test Kit Model FF-1A, Nine-Parameter Test Kit. Water quality parameters maintained throughout the experiment are in Table 5. To limit algal growth, tanks with nitrite and ammonia parameters outside of the acceptable range were scraped with an ethanol rinsed razor blade and water/debris was gravel vacuumed out to 50%. Water was then replaced with primed water prepared in XGSC.

At two months of age, Mini- Bacto-Surge Foam Filters (Hikari Aquarium Solutions; USA) were added to each tank to help maintain water quality. At one time point approximately three months into the experiment, 380 µL of API algae fix (API;

California) was added to all tanks to control for green discoloration that was hindering observation.

SEXING, WET WEIGHTS, STANDARD LENGTH, BODY WIDTH, BODY CONDITION FACTOR

Anesthetic solution was prepared using a buffered 1% MS-222 solution diluted to 0.01% with deionized water. Fish were separated and sexed by examination of the anal fin for platy fish and the dorsal and anal fins for medaka. Once sex was recorded, fish were placed into dishes with 4 mL of prepared anesthetic solution. A transfer pipette was used to aspirate the anesthetic solution out of petri dish and a Kim wipe used to dry remaining liquid in petri dish. Fish were weighed and moved into a six-well assay plate placed over millimeter grid paper. Fish were photographed over grid paper for later determination of standard body length and width using Image J software.

PLATY FISH BIRTH DATA

Beginning at 3 months of age, at 0730 h, platy fish tanks were inspected for fry daily. A skewer was used to agitate java moss and rocks in the tank for hidden young. Fry were then transferred to a watch glass separated by tank number and monitored for survival for 14 days.

MEDAKA EGG COLLECTION DATA

Beginning at 3 months of age, 30 minutes after feeding medaka, egg collections were conducted. When a female with eggs was observed, they were netted using a fine mesh brine net and the abdomen was gently massaged to remove the eggs. Number of eggs was recorded on an individual basis.

Eggs were inspected for removal of chorionic threads and fertilization status. Fertilized eggs were then transferred to a single petri dish per diet with 40 mL of embryo rearing solution. Embryo rearing solution was changed every other day and unfertilized or dead eggs were removed daily. Eggs then hatched in 7-14 days and hatchlings were transferred to a watch glass separated by diet to monitor survival for 14 days.

CALCULATIONS

Growth measures (weight, total length, and width) were expressed as percent change between timepoints: ((final value / initial value) * 100). Body condition factor was calculated as: ((wet weight (g) / (total length $(cm)^3$)) * 100) (Jones et al., 1999).

Reproductive measures were recorded as number of eggs collected, number of eggs hatched, and number of hatchlings that survived. Percent of eggs hatched was calculated as: ((number of eggs hatched / number of eggs collected) * 100). Survival rate of hatchlings was calculated as: ((number of hatchlings survived / number of eggs hatched) * 100).

Survival rate was calculated as: ((final number of fish in tank / (initial number of

fish in tank – fish sacrificed)) * 100).

ANALYSIS OF DATA

Statistical analysis compared differences in growth, reproduction, and survival between three different dietary treatments in two fish species over a six-month time period. This analysis was conducted in RStudio Statistical Software Version 1.3.1093 (R Core Team, 2013). Weight, total length, width, BCF, and reproductive data were organized in spreadsheets by tank, diet, and month. Averages were calculated for each diet combining tanks as replicates for each diet at each time point. Data were sorted in separate spreadsheets by timepoint and dietary treatment and saved as individual .csv files for analyses.

Data were analyzed using mixed model ANOVA with diet as a fixed factor and tanks nested as random factors within diet. Prior to each statistical analysis, a Shapiro-wilks normality test was run with the 'stats' package on the overall data with an alpha set at 0.01. If data had a normal distribution ($P > 0.01$), a parametric ANOVA test was performed using 'stats' package. *Post hoc* analyses and pair-wise analyses were conducted using *t*-tests. Non-normally distributed data ($P \leq 0.01$) were evaluated using the Kruskal Wallis rank sum test using the 'stats' package. In case of significant results, *post hoc* testing using the Nemenyi tests for multiple comparisons of rank sums with the 'PMCMR' package was completed (Pohlert, 2015). Results were organized into bar plots using the 'sciplot' package (Morales et al., 2012).

CHAPTER III. RESULTS

ANECDOTAL OBSERVATIONS

Differences in feed characteristics were observed among the dietary treatments throughout the 6-month study. CON and GEM feed floated on top of the water and sank over time while WAT feed dispersed throughout the water and quickly sank to the bottom of tank. The CON diet also consisted of live *Artemia* which dispersed upon contacting the water; both platy fish and medaka actively sought these out.

Medaka readily consumed all feeds, floating or sinking. However, platy fish only readily consumed CON feed. Platy fish also sought out live *Artemia* and the BLP of the CON diet, which slowly sank and was consumed before reaching the bottom. Excess GEM and WAT feed would be removed from platy fish tanks daily using a rinsed brine net 30 minutes after feeding.

Platy fish fed WAT and GEM diet were observably smaller, with duller scales, and moved less vigorously throughout the water column. Medaka fish fed WAT and GEM diet also appeared smaller and presented with more deformities such as spinal curvatures. Overall fish fed WAT and GEM diet appeared less healthy than fish fed CON diet. Water quality seemed to deteriorate quicker in WAT tanks and became difficult to visualize fish at certain points of the experiment.

GROWTH: PLATY FISH

Differences were observed in baseline measures of weight [$F(5) = 3.9$, $P \leq 0.01$] and length [$F(5) = 3.9$, $P \leq 0.05$] but not width [$F(5) = 3.9$, $P = 0.09$] for platy fish. At baseline, WAT fish (81.2 ± 14.8 mg) were significantly heavier [$F(5) = 3.9$, $P \leq 0.01$] than GEM (35.1 ± 5.5 mg) or CON (47.5 ± 24.4 mg). There was not a difference between weight of CON and GEM fish [$F(5) = 3.9$, $P = 0.57$]. WAT fish were significantly longer than GEM [$F(5) = 3.9$, $P \leq 0.05$], 17.0 ± 2.0 mm versus 12.3 ± 0.9 mm, respectively. There was not a difference in length between CON and GEM [$F(5) = 3.9$, $P = 0.50$] or CON and WAT fish [$F(5) = 3.9$, $P = 0.17$].

To account for differences in growth measurements at baseline, we expressed the weight, length, and width measurements as percent change from baseline (1 month of age) to juvenile (3 months of age) or baseline to mature (6 months of age) (Figure 2). Diet affected the percent change in weight of platy fish (Table 6). From baseline to 3 months of age, the percent change in weight of CON fish was 905 ± 357%, which was significantly greater [$\chi^2(2) = 9.8$, $P \leq 0.01$] than WAT (43 ± 15%), and trended [$\chi^2(2) = 9.8$, $P = 0.06$] to be more than GEM (145 ± 43%). Similarly, from baseline to 6 months of age, the percent change in weight of CON fish was 1894 ± 796% which was significantly [$\chi^2(2) = 9.8$, $P \leq 0.01$] greater than WAT (98 ± 53%) and trended [$\chi^2(2) = 9.8$, $P = 0.06$] to be more than GEM (701 ± 374%). There was a trend for a difference between GEM and WAT [$\chi^2(2) = 9.8$, $P = 0.06$].

Diet affected the percent change in length of platy fish (Table 6). From baseline to 3 months of age, the percent change in length of CON was 136 ± 37%, which was

significantly greater [χ^2 (2) = 9.8, $P \leq 0.01$] than for WAT (22 ± 4%), and trended [χ^2 (2) = 9.8, $P = 0.06$] to be greater than GEM (56 ± 10%). The difference between GEM and WAT was a statistical trend [χ^2 (2) = 9.8, $P = 0.06$]. From baseline to 6 months of age, the percent change in length of CON fish was 194 ± 63%, which was significantly [χ^2 (2) = 8.8, $P \leq 0.01$] greater than WAT (48 ± 5%), but not different than GEM (128 ± 27%) [χ^2 (2) = 8.8, $P = 0.12$]. The difference between GEM and WAT was statistically significant [χ^2(2) = 8.8, $P \leq 0.05$] with the percent change in length for GEM being greater.

Diet affected the percent change in width of platy fish (Table 6). From baseline to 3 months of age, the percent change in width of CON was 109 ± 25%, which was significantly greater [χ^2(2) = 8.8, $P \leq 0.01$] than for WAT (7 ± 9%) and GEM (27 ± 19%). GEM and WAT were similar [χ^2(2) = 8.8, $P = 0.12$]. From baseline to 6 months of age, the percent change in width of CON was 146 ± 45%, which was significantly [χ^2(2) = 9.3, $P \leq 0.01$] greater than WAT (16 ± 10%), and trended [χ^2(2) = 9.3, $P = 0.08$] to be greater than GEM (79 ± 29%). The percent change in width was greater [χ^2(2) = 9.3, $P \leq 0.05$] for GEM than for WAT.

Body condition factor was also calculated to assess nutritional status through length-weight models (Jones et al., 1999). Diet did not significantly affect BCF in Xiphophorus at baseline [χ^2(2) = 0.81, $P = 0.67$], 3 months of age [χ^2(2) = 0.46, $P = 0.79$], or 6 months of age [χ^2(2) = 4.15, $P = 0.13$] (Table 7).

REPRODUCTION: PLATY FISH

In platy fish, live births were only observed for CON. During month six, 21 fry were collected from CON tanks, separated, and raised for 2 weeks for survivability, which was 100%.

SURVIVAL: PLATY FISH

Overall, there was a difference between survival rate for platy fish [$F(5) = 3.9$, $P \leq 0.05$] (Table 11, Figure 7). CON had a 95 ± 9% survival rate which was significantly higher [$F(5) = 3.9$, $P \leq 0.05$] than GEM (67 ± 13%) but not significantly different [$F(5) = 3.9$, $P = 0.15$] from WAT (76 ± 16%). No differences were observed between GEM and WAT [$F(5) = 3.9$, $P = 0.42$].

GROWTH: MEDAKA

Differences were observed in baseline measures of length [$F(5) = 3.9$, $P \leq 0.05$] and width [$F(5) = 3.9$, $P \leq 0.05$], but not weight [$F(5) = 3.9$, $P = 0.37$] for medaka. At baseline, WAT fish (15.9 ± 0.5 mm) were significantly longer [$F(5) = 3.9$, $P \leq 0.05$] than GEM (13.6 ± 1.4 mm) and CON fish (15.3 ± 0.7 mm) trended to be longer than GEM [$F(5) = 3.9$, $P = 0.08$]. There was not a difference in length between CON and WAT [$F(5) = 3.9$, $P = 0.65$]. WAT (2.5 ± 0.1 mm) and CON fish (2.5 ± 0.2 mm) were significantly wider [$F(5) = 3.9$, $P \leq 0.05$] than GEM (2.1 ± 0.2 mm). No differences were observed in width between CON and WAT fish [$F(5) = 3.9$, $P = 0.98$].

To account for differences in baseline data, changes in growth measurements

were calculated as percent change from baseline (1 month of age) to juvenile (3 months of age) and mature (6 months of age) (Figure 3). Diet affected the percent change in weight of medaka (Table 8). From baseline to 3 months of age, the percent change in weight of CON fish was 319 ± 32% , which was significantly greater [$F(5) = 3.9$, $P \leq 0.01$] than for GEM (206 ± 44%) or WAT (156 ± 23%). There was not a statistical difference between GEM and WAT [$F(5) = 3.9$, $P = 0.15$]. From baseline to 6 months of age, the percent change in weight of CON (674 ± 60%) and GEM (634 ± 166%) was significantly greater [$F(5) = 3.9$, $P \leq 0.01$] than WAT (396 ± 90%) with no statistical difference [$F(5) = 3.9$, $P = 0.87$] between CON and GEM.

Diet affected the percent change in length of medaka (Table 8). From baseline to 3 months of age, the percent change in length of CON (90 ± 15%) and GEM (103 ± 29%) was significantly greater [$F(5) = 3.9$, $P \leq 0.05$] than that of WAT (47 ± 14%). The percent change in length between CON and GEM fish was not statistically different [$F(5) = 3.9$, $P = 0.65$]. From baseline to 6 months of age, the percent change in length of GEM (157 ± 37%) was significantly greater [$F(5) = 3.9$, $P \leq 0.05$] than WAT (101 ± 21%), but not statistically different from CON (137 ± 19%) [$F(5) = 3.9$, $P = 0.56$].

Diet did not significantly affect the percent change in width of medaka (Table 8). From baseline to 3 months of age, the percent change in width of CON (38 ± 15%) and GEM (50 ± 21%) was statistically similar [$F(5) = 3.9$, $P = 0.56$]. GEM fish trended to be wider than WAT (19 ± 14%)[$F(5) = 3.9$, $P = 0.06$]. From baseline to 6 months of age, the percent change in width of GEM (119 ± 312%) and CON (118 ± 53%) trended [$F(5) = 3.9$, $P = 0.08$] to be wider than WAT (55 ± 12%), but GEM and CON were not statistically different from each other [$F(5) = 3.9$, $P = 0.99$].

Diet significantly affected BCF in medaka (Table 9). There were no statistical differences at baseline. However, at 3 months of age, GEM (0.57 ± 0.09) had a statistically different [χ^2 (2)= 6.6, P ≤ 0.05] BCF from WAT (0.91 ± 0.20) but was statistically similar to CON (0.80 ± 0.12). At 6 months of age, no statistical differences were observed in BCF.

REPRODUCTION: MEDAKA

Diet impacted the number of eggs collected for medaka (Table 10, Figure 4). Number of eggs collected per diet was not significantly different overall [χ^2(2) = 3.7, *P* = 0.16] but there were differences between diets. Significantly more eggs [χ^2(2) = 3.7, *P* ≤ 0.05] were collected for CON (76.6 ± 70.5) versus GEM (16 ± 13.4) and there was a trend [χ^2(2)= 3.7, *P* = 0.06] for more eggs to be collected from CON versus WAT (18.4 ± 16.2). There were no differences between GEM and WAT [χ^2(2) = 3.7, *P* = 0.40].

Diet did not impact percent of eggs hatched [χ^2(2) = 0.75, *P* = 0.69] (Table 10, Figure 5). The hatching rate for CON was 53 ± 11%, for GEM was 43 ± 40%, and for WAT was 38 ± 25%. Diet impacted the survival rate of hatched eggs between diets, although there were not significantly overall differences [χ^2 (2) = 4.1, *P* = 0.13] (Table 10, Figure 6). There was a significantly higher [χ^2(2) = 4.1, *P* ≤ 0.05] hatchling survival rate for CON diet (87 ± 9%) versus GEM (47 ± 43%) and a trend [χ^2 (2)= 4.1, *P* = 0.14] for CON to be higher than WAT (60 ± 42%). No differences were observed between GEM and WAT [χ^2 (2)= 4.1, *P* = 0.17].

SURVIVAL: MEDAKA

There was a trend for an overall difference in survival rate between diets for medaka [χ^2 (2) = 5.2, P = 0.07] (Table 11, Figure 7). CON had a 100% survival rate which was significantly higher [χ^2(2) = 5.2, $P \leq 0.01$] than GEM (91 ± 3%) and WAT (91 ± 2%). Survival rate for GEM and WAT was not significantly different [χ^2(2) = 5.2, P = 0.42].

CHAPTER IV. DISCUSSION

This study evaluated the effects of three diets on platy fish and medaka growth, reproduction, and survival rate using: 1) a diet formulated specifically for the XGSC (CON); 2) a commercially available zebrafish diet (GEM); and 3) a laboratory defined zebrafish reference diet (WAT). Significant differences were observed in growth, reproduction, and survival of both species of fish despite controlled feeding protocols and other environmental factors.

Commercial feeds used to culture zebrafish that are bred specifically for research vary widely in their published concentrations of major dietary constituents (Siccardi et al., 2009). Unlike zebrafish, medaka and platy fish do not have defined reference diets. The diets tested in this study (CON, GEM, and WAT) are all formulated and marketed for laboratory fish; however, when fed under controlled conditions, resulted in significant differences in outcomes for two commonly used laboratory fish, platy fish and medaka.

Diets used in this study varied in terms of nutritional value. Crude protein ranged from 43 to 51% and crude fat from 9 to 21%. Minerals and vitamins, feed ingredient sources, and trace elements also varied. Provision of live versus flake feed is also a key difference. Cumulatively, these differences are likely responsible for our observations of differences in growth and reproductive parameters. These data demonstrate that, even when feeding protocols are identical, dietary-mediated differences in performance outcomes occur. These differences hinder reproducibility of experiments, making inter-laboratory comparisons difficult.

A recent article by Fowler et al. (2019) demonstrated similar results to ours in

zebrafish. They reported excellent growth profiles and survival rates for all diets tested; however, there were significant differences in terminal weight gain, BCF, body fat deposition, and reproductive outcomes between dietary treatments.

Growth outcomes represent a fish's response to nutrition (Fowler et al., 2019). Fish were grown throughout the course of our study from the late larval, to juvenile, to mature adult stage. The nutrient and ingredient variance inherent in commercially available diets could influence growth in the somatic phase and limit reproductive capabilities. Dietary fatty acids play an essential role in reproductive outcomes in fish (Araujo et al., 2016). The high levels of essential fatty acids provided by *Artemia* and beef liver paste could account for the reproductive success of platy fish fed CON versus those fed GEM or WAT that did not produce fry. Additionally, for medaka, fish fed CON had the most eggs collected and hatchlings survived.

Some diets are labeled for a specific life stage (larval, juvenile, or mature) and others are for all life stages. However, as dietary requirements change throughout life, so should the diet. In the study conducted by Fowler et al. (2019), zebrafish fed only *Artemia* had an increased growth advantage in the first six weeks of the feeding trial compared to commercial flake and a formulated reference diet. Similarly, platy fish and medaka in our study had significantly greater percent changes in growth measures from baseline to juvenile life (3-months of age) when fed the CON diet (Aquatox with *Artemia*) versus the other diets (no live feed). However, in the study conducted by Fowler et al. (2019), the *Artemia*-fed zebrafish fell behind, in terms of growth, after the juvenile phase while, in our study, CON fed fish continued to have greater percent change in weight versus WAT in medaka and in percent change in weight, length, and width versus

WAT and GEM in platy fish.

Wild zebrafish, such as the populations in south Asia, consume zooplankton, insects, and various plant matter (Spence et al., 2007). This multisource diet most closely reflects the CON diet in our study. In platy fish, the CON diet consistently resulted in better growth than either the WAT or GEM diets at both the juvenile and mature life stages (Figure 2). This effect was also present in medaka, but to a lesser extent, as only weight was impacted versus the GEM and WAT diets (Figure 3). Ultimately, medaka and platy fish are omnivorous fish that would feed on similar diets as zebrafish in the wild and may benefit from multisource diets if these diets are able to be managed and controlled such that they are standardized and consistent.

Nutritional status of fish can also be assessed through length-weight models defined as BCF (Jones et al., 1999). BCF, determined by estimated weight or length of the fish, is used to determine optimal daily feed rations for different life stages of the fish (Jones et al., 1999). Additionally, BCF is crucial for determining the health of a population and can alert a fish culturist to the onset of disease, stress due to overcrowding, or other physiological effects before high mortality rates are endured (Jones et al., 1999).

Accordingly, the use of BCF in this study allowed for a length-weight visual to determine differences in diets. In platy fish, no differences were observed in BCF at baseline, 3 months, or 6 months. These results beg the question as to whether BCF is actually a better representation of fish health when compared to actual changes in length, weight, and width of the fish as survival rate of the platy fish was significantly different between treatments as well as anecdotal observations that GEM and WAT platy fish

appeared to be less healthy than CON.

In medaka, WAT had a significantly greater BCF than GEM at 3 months and there were no other differences between diets at this or other timepoints. A high BCF may be the result of overconsumption of a specific nutrient from a commercial or defined diet and can reflect negative health outcomes similar to high body mass index (BMI) in humans (Fowler et al., 2019). A high BMI can result in obesity and other health associated problems which could be associated with the lower survival rate in WAT diet (Figure 7).

Medaka become reproductively mature and are essentially full grown around three to four months of age (Denny et al., 2004). Being the most taxing period of the life in terms of growth and reproduction, the numerically lower BCF in GEM fish at the 3-month timepoint may indicate undernutrition in the juvenile life stage and may explain the low number of eggs collected and percent survival of hatchlings in the GEM diet. It is worth noting that BCF is traditionally used as a health indicator in wild fish populations but has not been thoroughly defined in laboratory fish; thus, these findings should be interpreted with caution (Fowler et al., 2019). There may be value in combining the use of BCF and other growth parameters to evaluate health and nutritional status of laboratory fish.

We must consider if our general observations, that fish consuming certain diets had differential effects on growth and reproduction, in addition to survivability of offspring, is rooted in nutritional programming (also referred to as metabolic programming). Nutritional programming is reflected through the documented correlation between suboptimal nutritional status during early life and predisposition to metabolic

diseases in later life (reviewed by Hou and Fuiman, 2020). More specifically, suboptimal maternal nutrition during pregnancy or during the larval stage could insult developing systems, causing long-term consequences. Programming effects in fish have been documented in growth, survival, neural development, and nutrient utilization (Chang et al., 2008; Collins et al., 2013; Engrola et al., 2018).

With regards to teleost species, a soybean meal diet at first feeding in rainbow trout (*Oncorhynchus mykiss*) improved the palatability and utilization of the same diet later in life (Geurden et al., 2013). In zebrafish (*Danio rerio*), early nutritional intervention altered carbohydrate digestion in later life (Fang et al., 2013). Additionally, gut function in zebrafish (*Danio rerio*) is demonstrably altered by early programming on a molecular level (Perera et al., 2016). The lack of growth in the early phases of life for GEM and WAT comparatively could have produced reduced egg production and survival rates later in life.

Overall, mature fish survivability rate was notably different between diets; survivability is an important outcome when raising laboratory animals for experimentation. Nutritional programming could have played a role in survival as all fish in our study were raised from birth to 1-month of age on *Artemia* and Aquatox flake and then changed overnight to either CON, GEM, or WAT diet. Anecdotally, we observed that platy fish did not readily consume the GEM or WAT diet as compared to CON and that likely resulted in early stunting, as reflected through our growth observations at later timepoints.

Collectively, our findings emphasize the need for laboratories to develop standardized feeding practices, report dietary data, and define reference diets based on

fish species. These data provide a foundation for the development of a defined, open-formulation diet for different species with a proposal for future dietary standardization of laboratory fish models. Our results demonstrate the importance of feeding defined diets and the potential experimental differences that may arise from the use of undefined or unstandardized diets. It is important to recognize that any differences in outcomes tested among the diets cannot be used to justify the efficacy of any individual diet; only to justify that differences were observed among the diets.

Notably, our fish being backgrounded on *Artemia* from birth to one month of age is a study limitation and this should be taken into consideration when interpreting data. The argument could be made that CON fish had an advantage because they were already familiar with part of the diet. Future studies striving to achieve reproducibility of our study should consider this. Future research should also consider feeding all commercial diets with a mix of live feed and documenting if this bridges the gap in growth and reproductive differences we observed rather than focusing on creating the same diet for all fish.

Table 1. Dietary Mediation of Growth in Fish

Reference	Fish species	Dietary treatment	Growth effect
Hu et al., 2013	Japanese seabass (*Lateolabrax japonicus*)	Protein manipulation (fish meal vs animal blend)	• Significant differences in Specific Growth Rate (SGR) between protein sources • Significant differences in whole body lipid concentrations
Ahmadifar et al., 2014	Great Sturgeon (*Huso huso*)	Essential oils added to basal diet	• Increased weight gain • Increased SGR • Increased Food Consumption Rate
Fang et al., 2013	Zebrafish (D*anio rerio*)	Carbohydrate manipulation	• Increased larval growth rates with higher carbohydrates (CHO) • Upregulation of key genes involved in glycolytic and pancreatic enzyme production with higher CHOs
Fowler et al., 2019	Zebrafish (*Danio rerio*)	Different commercially available fish diets	• Significant differences in growth and body composition

Table 2. Dietary Mediation of Reproduction in Fish

Reference	Fish species	Dietary treatment	Growth effect
Gonzales, 2012	Zebrafish (*Danio rerio*)	Different commercially available fish diets	• Significantly different spawning success • Significantly different fecundity rates
Fowler et al., 2019	Zebrafish (*Danio rerio*)	Different commercially available fish diets	• Significant differences in Gonadosomatic Index (GSI) • Significantly different successful spawning • Significantly different embryo viability
Kowalska et al., 2020	Medaka (*Oryzias latipes*)	Diet supplemented with PUFA	• Significantly higher fertility rates • Significantly higher sperm motility • Increase in embryo survival rates
Martin et al., 2021	Senegalese sole (*Solea senegalensis*)	Natural feeding vs. commercial feeding	• Significantly different sperm production • Significantly different sperm motility • Significantly different GSI

Table 3. Proximate Analysis of Diets						
	Treatment[a]					
		CON		GEM	WAT	
	BLP[b]	*Artemia*	Aquatox		Juvenile	Adult
Protein, %	13.6	62.8	51.8	60.1	43.1	44.2
Crude Fat, %	8.3	6.2	11.8	18.9	22.0	9.5
Moisture, %	-	6	3.3	6.4	8.5	7.9
Nitrogen, %	-	-	8.3	9.6	6.9	7.1
Ash, %	-	4.9	11.2	12.4	5.2	14.9
Crude Fiber, %	2.2	0.6	0.6	0.40	2.8	2.8
Metabolizable Energy, kcal/lb	-	-	1,550	1,592	1,760	1,366
Digestible Energy, kcal/lb	-	-	1,810	1,896	2,014	1,567
NFE, Calculated	-	-	21.34	1.9	18.4	20.7

[a]CON = beef liver paste (BLP), *Artemia*, and Ziegler Aquatox flake; GEM = Skretting Gemma Micro150; WAT = laboratory-defined reference diet developed by Steven Watts at University of Alabama Birmingham

[b]Proximate analysis conducted by Eurofins Inc. for all ingredients except for BLP, in which values were estimated

Table 4. Elemental Analysis of Diets					
	Treatment[a]				
	CON		GEM	WAT	
	BLP[b]	Aquatox		Juvenile	Adult
Calcium, %	0.25	2.41	1.72	0.48	0.49
Iron, %	0.02	0.02	0.03	0.01	0.05
Magnesium, %	0.02	0.17	0.25	0.08	0.10
Phosphorous, %	0.11	1.45	1.96	0.08	0.83
Potassium, %	0.18	0.79	1.02	0.09	0.80
Sodium, %	0.61	0.71	1.04	0.61	0.63
Sulfur, %	-	0.61	1.48	0.43	0.43
Copper, ppm	0.9	11	19	6.1	8.4
Manganese, ppm	-	41	60	9.8	12
Zinc, ppm	27	126	202	46	47
[a]CON = beef liver paste (BLP), *Artemia*, and Ziegler Aquatox flake; GEM = Skretting Gemma Micro150; WAT = laboratory-defined reference diet developed by Steven Watts at University of Alabama Birmingham [b]Elemental analysis conducted by Eurofins Inc. for all ingredients except for BLP, in which values were estimated					

Table 5. Water Chemistry		
Parameter	Value[a]	Testing Frequency
Alkalinity, mg/L $CaCO_3$	284.7 ± 49.6	Monthly
Ammonia, mg/L NH_3	0.01 ± 0.02	Bi-Weekly
Carbon dioxide, mg/L CO_2	7.3 ± 4.1	Monthly
Dissolved oxygen, mg/L O_2	8.3 ± 1.4	Monthly
Nitrite, mg/L	0.2 ± 0.3	Bi-Weekly
pH	8.4 ± 0.2	Monthly
[a]Values are presented as Mean ± SEM		

Table 6. Percent Change in Platy Fish Growth by Diet

	Treatment[a]			*P*-value	Contrast (*P*-value)		
	CON[b]	GEM	WAT		CON vs GEM	CON vs WAT	GEM vs WAT
Weight, % change[c]							
Baseline to 3 mo.	905 ± 357	145 ± 43	43 ± 15	≤0.01	0.06	≤0.01	0.06
Baseline to 6 mo.	1894 ± 796	701 ± 374	98 ± 53	≤0.01	0.06	≤0.01	0.06
Length, % change							
Baseline to 3 mo.	136 ± 37	56 ± 10	22 ± 4	≤0.01	0.06	≤0.01	0.06
Baseline to 6 mo.	194 ± 63	128 ± 27	48 ± 5	≤0.01	0.12	≤0.01	0.04
Width % change							
Baseline to 3 mo.	109 ± 25	27 ± 19	7 ± 9.4	≤0.01	0.04	≤0.01	0.12
Baseline to 6 mo.	146 ± 45	79 ± 29	16 ± 10	≤0.01	0.08	≤0.01	0.05

[a]CON = beef liver paste (BLP), *Artemia*, and Ziegler Aquatox flake; GEM = Skretting Gemma Micro150/300; WAT = laboratory-defined reference diet developed by Steven Watts at University of Alabama Birmingham
[b]Values are presented as Mean ± SEM
[c]Percent change calculated as: ((final value / initial value) * 100)

Table 7. Platy Fish Body Condition Factor (BCF) by Diet

	Treatment[a]			
	CON[b]	GEM	WAT	*P*-value
BCF[c]				
Baseline	1.63 ± 0.18	1.97 ± 0.63	1.71 ± 0.47	0.67
3 months	1.26 ± 0.35	1.23 ± 0.12	1.31 ± 0.17	0.79
6 months	1.29 ± 0.27	1.19 ± 0.11	1.00 ± 0.14	0.13

[a]CON = beef liver paste (BLP), *Artemia*, and Ziegler Aquatox flake; GEM = Skretting Gemma Micro150/300; WAT = laboratory-defined reference diet developed by Steven Watts at University of Alabama Birmingham
[b]Values are presented as Mean ± SEM
[c]BCF calculated as: ((Wet weight (g) / (total length $(cm)^3$)) * 100)

Table 8. Percent Change in Medaka Growth by Diet

	Treatment[a]			*P*-value	Contrast (*P*-value)		
	CON[b]	GEM[b]	WAT[b]		CON vs GEM	CON vs WAT	GEM vs WAT
Weight, % change[c]							
Baseline to 3 mo.	319 ± 32	206 ± 44	156 ± 23	≤0.01	≤.01	≤0.01	0.15
Baseline to 6 mo.	674 ± 60	634 ± 166	396 ± 90	≤0.01	0.87	≤0.01	0.03
Length, % change							
Baseline to 3 mo.	90 ± 15	103 ± 29	47 ± 14	≤0.01	0.65	0.03	≤0.01
Baseline to 6 mo.	137 ± 19	157 ± 37	101± 21	≤0.05	0.56	0.20	0.04
Width % change							
Baseline to 3 mo.	38 ± 15	50 ± 21	19 ± 14	≤0.05	0.56	0.29	0.06
Baseline to 6 mo.	118 ± 53	119 ± 312	55 ± 12	≤0.05	0.99	0.08	0.07

[a]CON = *Artemia*, and Ziegler Aquatox flake; GEM = Skretting Gemma Micro150/300; WAT = laboratory-defined reference diet developed by Steven Watts at University of Alabama Birmingham
[b]Values are presented as Mean ± SEM
[c]Percent change calculated as: ((final value / initial value) * 100)

Table 9. Medaka Body Condition Factor (BCF) by Diet

	Treatment[a]			
	CON[b]	GEM	WAT	*P*-value
BCF[c]				
Baseline	1.29 ± 0.17	1.56 ± 0.36	1.13 ± 0.35	0.25
3 months	0.80 ± 0.12	0.57 ± 0.09	0.91 ± 0.20	0.03[d]
6 months	0.76 ± 0.09	0.68 ± 0.17	0.69 ± 0.21	0.58

[a]CON = *Artemia*, and Ziegler Aquatox flake; GEM = Skretting Gemma Micro150/300; WAT = laboratory-defined reference diet developed by Steven Watts at University of Alabama Birmingham
[b]Values are presented as Mean ± SEM
[c]BCF calculated as: ((Wet weight (g) / (total length $(cm)^3$)) * 100)
[d]Contrast *P*-Value: CON-GEM $P = 0.26$; CON-WAT $P = 0.59$; GEM-WAT $P \leq 0.05$

Table 10. Reproduction Outcomes in Medaka by Diet							
	Treatment[a]			*P*-value	Contrast (*P*-value)		
	CON[b]	GEM	WAT		CON vs GEM	CON vs WAT	GEM vs WAT
No. of Eggs	76.6 ± 70.5	16 ± 13.4	18.4 ± 16.2	0.16	0.04	0.06	0.40
Hatched, %[c]	53 ± 11	43 ± 40	38 ± 25	0.69	0.50	0.23	0.23
Survived, %[d]	87 ± 9	47 ± 43	60 ± 42	0.13	0.02	0.14	0.17

[a]CON = *Artemia*, and Ziegler Aquatox flake; GEM = Skretting Gemma Micro150/300; WAT = laboratory-defined reference diet developed by Steven Watts at University of Alabama Birmingham
[b]Values are presented as Mean ± SEM
[c]Percent eggs hatched calculated as: ((Number of eggs hatched / Number of eggs collected) * 100)
[d]Percent survival of hatchlings calculated as: ((number of hatchlings survived / number of eggs hatched) * 100)

Table 11. Survival Rate of Platy Fish and Medaka Over a Six-Month Time Period by Diet							
	Treatment[a]			*P*-Value	Contrast (*P*-value)		
	CON[b]	GEM	WAT		CON vs GEM	CON vs WAT	GEM vs WAT
Platy fish, %[c]	95 ± 9	67 ± 13	76 ± 16	≤ 0.05	0.03	0.15	0.63
Medaka, %	100 ± 0	91 ± 3	91 ± 2	≤ 0.05	≤ 0.01	≤ 0.01	0.42

[a]CON = beef liver paste (BLP) (excluded for medaka), *Artemia*, and Ziegler Aquatox flake; GEM = Skretting Gemma Micro150/300; WAT = laboratory-defined reference diet developed by Steven Watts at University of Alabama Birmingham
[b]Values are presented as Mean (SEM)
[c]Percent survival rate calculated as: ((final number of fish in tank / (initial number of fish in tank – fish sacrificed)) * 100)

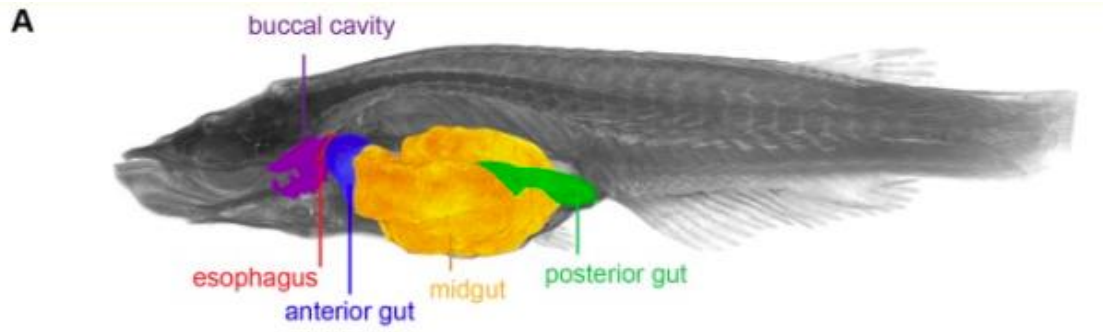

Figure 1. 3D image of adult medaka taken by X-ray microCT (Aghaallaei et al., 2016)

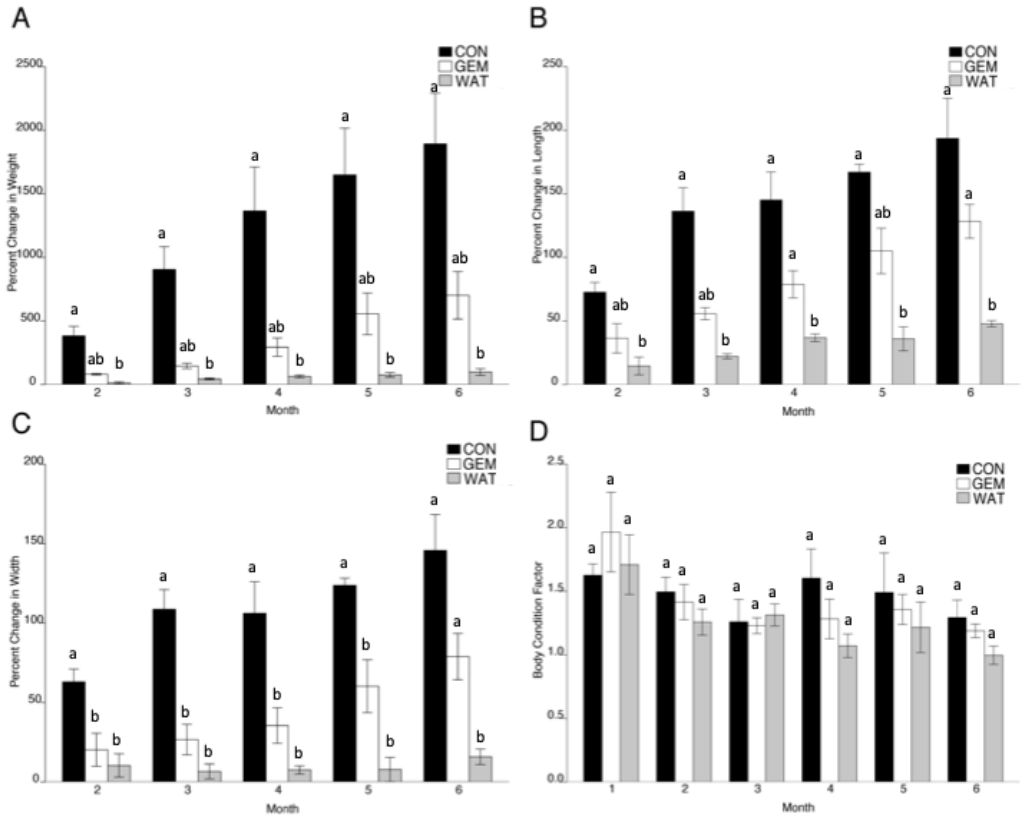

Figure 2. Growth Outcomes in Platy Fish. Groups with different letters are significantly different at $P \leq 0.05$; A) Percent change in weight of platy fish over six months by dietary treatment; B) Percent change in length of platy fish over six months by dietary treatment; C) Percent change in width of platy fish over six months by dietary treatment D) Body condition factor of platy fish compared at each month by dietary treatment; Percent change calculated as: ((Final value / Initial value) * 100); Body condition factor calculated as: ((Wet weight (g) / (total length (cm)3)) * 100)

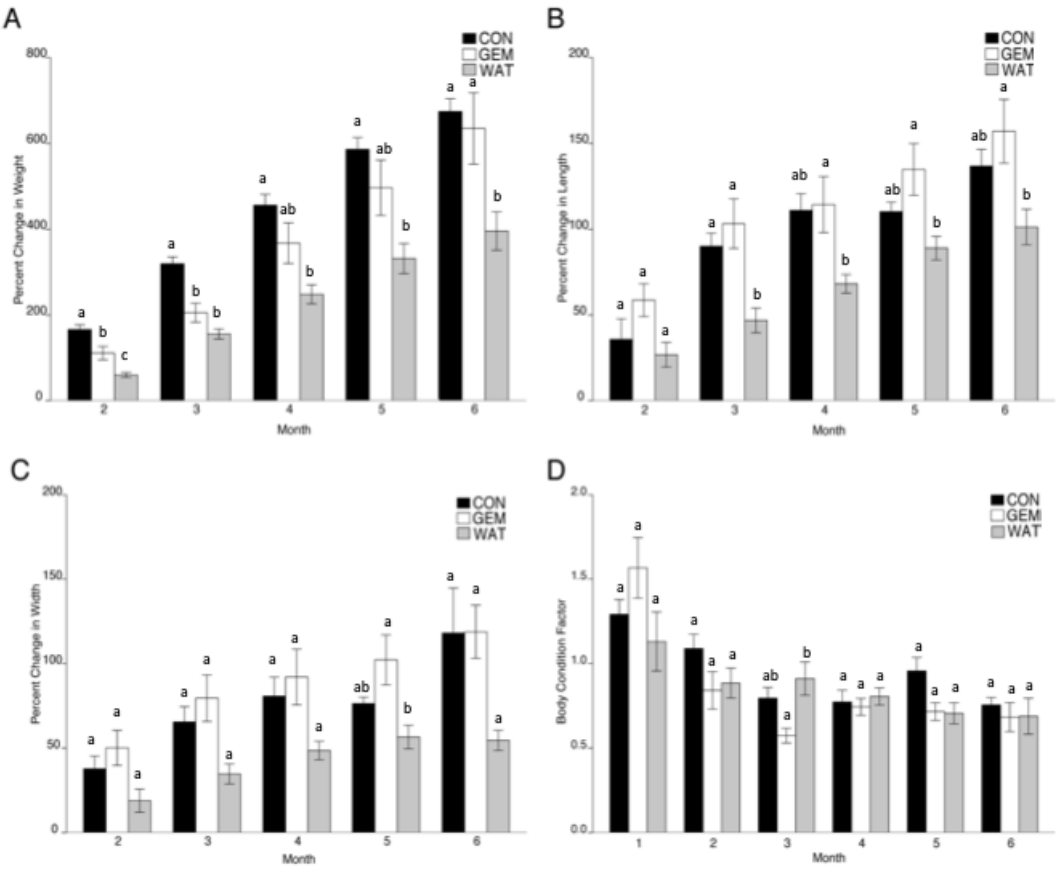

Figure 3. Growth Outcomes in Medaka. Groups with different letters are significantly different at $P \leq 0.05$; A) Percent change in weight of medaka over six months by dietary treatment; B) Percent change in length of medaka over six months by dietary treatment; C) Percent change in width of medaka over six months by dietary treatment D) Body condition factor of medaka compared at each month by dietary treatment; Percent change calculated as: ((Final value / Initial value) * 100); Body condition factor calculated as: ((Wet weight (g) / (total length $(cm)^3$)) * 100)

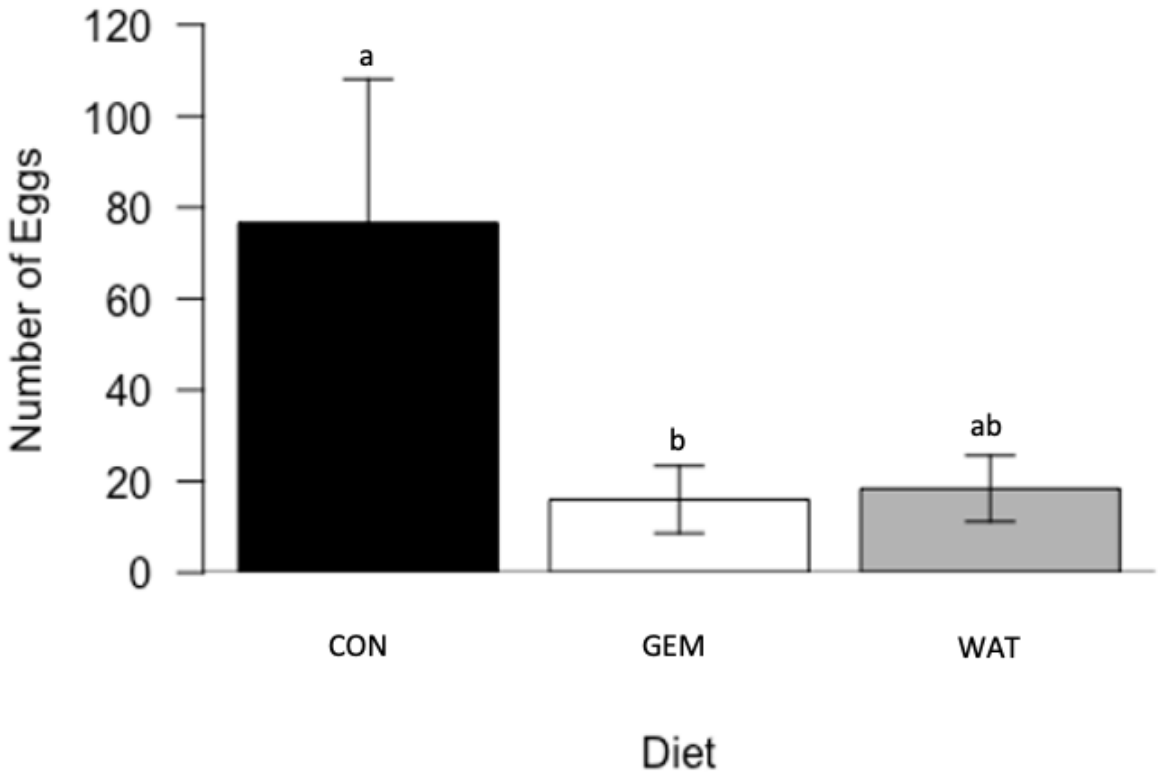

Figure 4. Total Number of Eggs Collected in Medaka by Diet

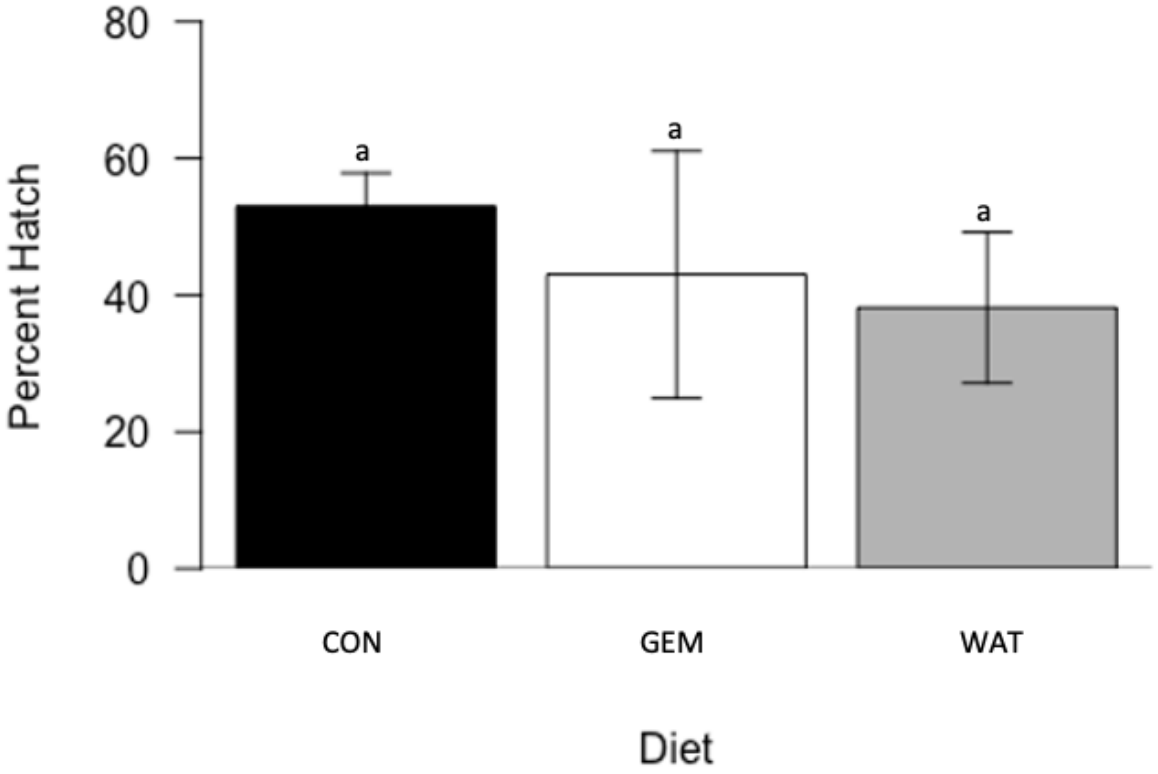

Figure 5. Percent of Eggs Hatched in Medaka by Diet
Percent of eggs hatched calculate as ((Number of eggs hatched / Number of eggs collected) * 100)

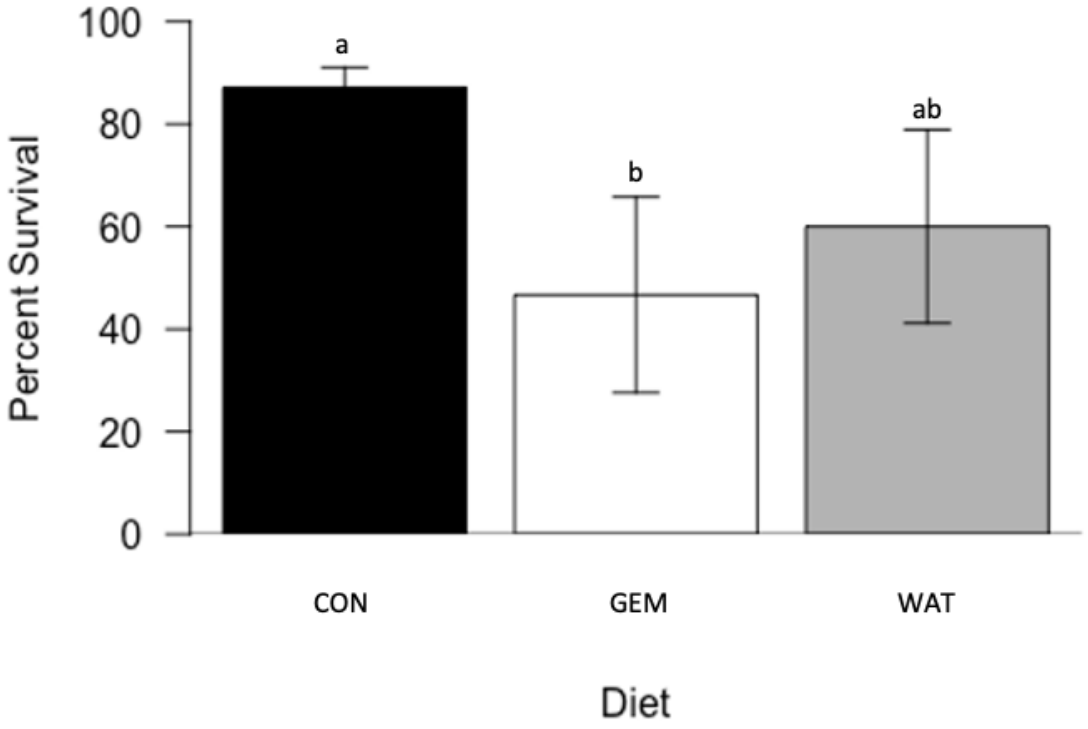

Figure 6. Percent Survival of Hatchlings Over Two Weeks in Medaka by Diet

Percent survival rate of hatchlings calculated as: ((Number of hatchlings survived / Number of eggs hatched) * 100)

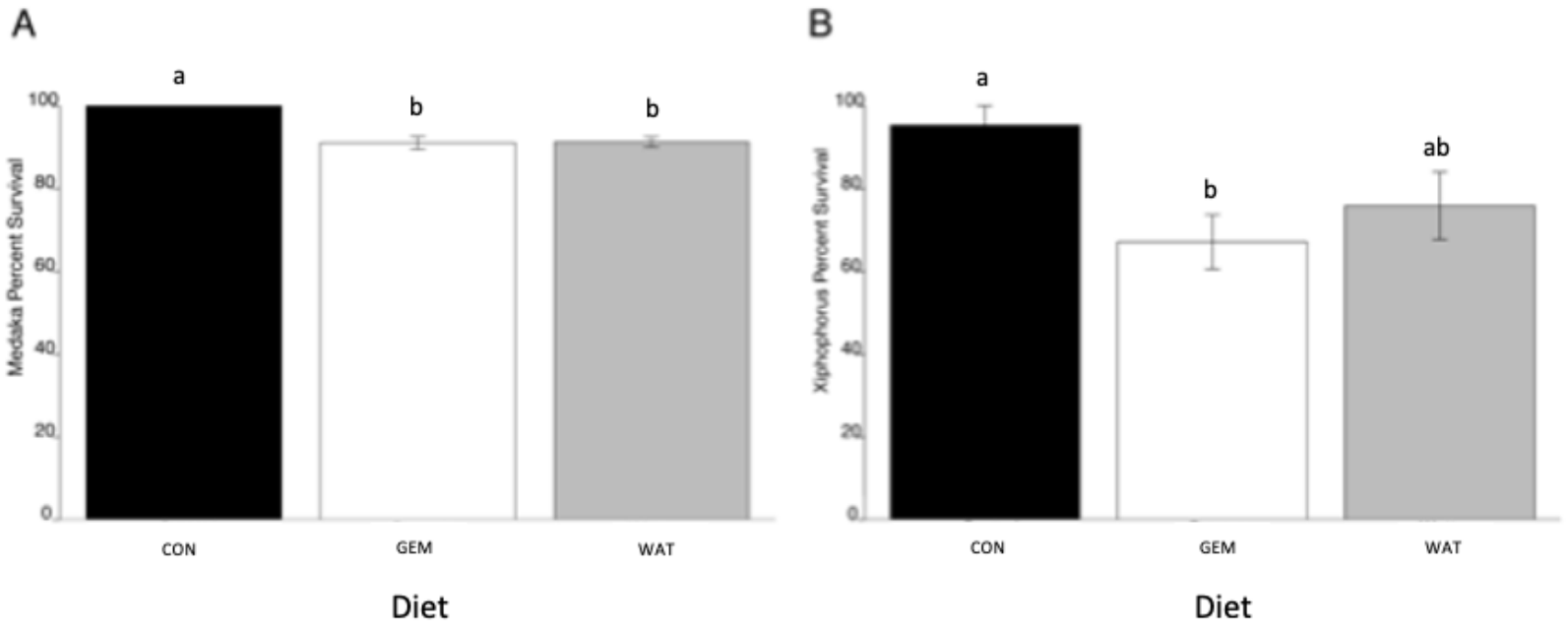

Figure 7. Survival Rate of Fish by Diet A) Survival rate of medaka over six month time period by diet; B) Survival rate of platy fish over six month time period by diet; Survival Rate calculated as: ((Final number of fish in tank / (initial number of fish in tank – fish sacrificed)) * 100)

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