COMBATING STRESS: THE USE OF ISOFLAVONES AS NEUTRACEUTICALS TO IMPROVE IMMUNITY AND GROWTH IN NILE TILAPIA (*OREOCHROMIS NILOTICUS*)

by

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To Peter M. Saya II, gone but not forgotten.

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ABSTRACT

Author: Furnas, Destin J. MS
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Title: Combating Stress: The Use of Isoflavones as Nutraceuticals to Improve Immunity and Growth of Nile Tilapia (*Oreochromis niloticus*)
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Stressors in the aquaculture environment can lead to negative impacts on growth and immune health, resulting in susceptibility to infectious diseases. These stressors are expected to increase as the growth of aquaculture continues to rise to meet demands for quality fish protein. Isoflavones, as a crude extract or as a pure isolate, may be effective in modulating the stress response, promoting growth and immunity. The objective of these studies was to examine the effect of various pure isoflavone isolates and crude isoflavone extracts on stress, growth, and immunity. Nile tilapia (*Oreochromis niloticus*) were stressed by adding hydrocortisone to the feed. In a 7-week study, pure isoflavone isolates of genistein and puerarin were evaluated to determine their respective effects on stress, growth, and immunity. A separate 10-day physiological and 6-week growth study focused on crude isoflavone extracts from kudzu (*Pueraria lobate*), red clover (*Trifolium pratense*), and soybean (*Glycine max*) was performed to determine their respective effects on stress, growth, and immunity. Numerous physiological parameters of the fish were measured (serum cortisol concentration, blood glucose concentration, hematocrit, hepatosomatic index, plasma protein concentration, lysozyme activity, and spleen-somatic index) to determine the effects of these pure isoflavone and crude isoflavone extracts on the modulation of stress and immunity. Many growth parameters were examined (length, weight, condition factor, weight gain, specific growth rate, feed intake, feed conversion ratio, and protein efficiency ratio) as well to determine the effects of these pure isoflavones and isoflavone extracts on growth. The addition of isoflavone and crude isoflavone extracts to the diet of Nile tilapia ameliorated some of the negative consequences of stress. Compared to stressed fish fed commercial feed, genistein and puerarin added to the diet appeared to improve serum cortisol concentrations, which resulted in increased plasma protein, albeit at different durations of stress. Puerarin, as well as all three crude isoflavone extracts, significantly increased spleen-somatic index compared to non-supplemented stressed fish, although the crude isoflavone extracts did

not appear to improve serum cortisol concentrations. Crude isoflavone extracts also showed overall increases in lysozyme activity compared to non-supplemented stressed fish, although this was not significant. Genistein, puerarin, and red clover showed increased growth rates, feed conversion ratio, and protein efficiency. Overall, pure isolates of isoflavone appear to be more effective in modulating stress, immunity, and growth than the crude isoflavone extracts, although red clover extract showed promises in the ability to modulate the stress response and improve growth and immunity. There are likely substantial interactions between the isoflavones in the crude extracts that cannot be fully understood by measuring the effects of single isoflavones. Regardless, isoflavone supplementation (pure or crude) appeared to generally have an overall positive impact on stressed Nile tilapia, requiring more research to better understand the effects and mechanisms behind these isoflavones.

GENERAL INTRODUCTION

Aquaculture, the production of aquatic plants and animals, is the fastest growing food sector in the world (Troell et al., 2014). With an average annual growth rate of 7.8% globally, aquaculture is far outcompeting other food sectors such as poultry, beef, and grain, which have annual growth rates of 4.6%, 1.0%, and 1.4%, respectively (Fig. 1). Since capture of natural fish populations began stagnating towards the end of the 20th century (Sumaila et al., 2016), aquaculture has been growing to meet those increased demands. Globally, the Asian-Pacific region accounts for a disproportionate production of fish via aquaculture, with China being the world's largest producing country (Little et al., 2016). Comparably, North America plays very little role in aquaculture production, accounting for less than one percent of global production (Harvey, 2017).

As global population continues to increase, the challenge of providing the world with a high-quality source of food will need to be addressed. Currently, growth in aquaculture production is surpassing population growth (Little et al., 2016). With that growth though, it is important to know who that increase in production is benefiting. So far, most of that growth has not come from necessity, but an increase in consumption from Western countries (Little et al., 2016). Meanwhile, caloric deficiency affects 800 million worldwide, with malnutrition being a substantial contributor to disease burden (IFPRI, 2016). Aquaculture has the opportunity to close this caloric deficiency gap and help feed a growing and hungry world. To have the greatest impact, selection of an appropriate species for cultivation should be considered.

Nile tilapia (*Oreochromis niloticus*) is currently a heavily cultivated fish in aquaculture and has the potential to meet these growing nutritional demands. Nile tilapia production in aquaculture has been rapidly growing since the 1980s (Fig. 2), making it the fifth most cultivated species in aquaculture, behind other species such as grass carp (*Ctenopharyngodon idellus*) and common carp (*Cyprinus carpio*) (FAO, 2018). There are numerous benefits to Nile tilapia cultivation in aquaculture: 1) efficient conversion of feed into quality protein, 2) low production of cost, and 3) high tolerance and adaptability. First, Nile tilapia has a relatively low feed conversion ratio (high feeding efficiency), approaching a near feed-in, feed-out system (de Silva, et al., 2017; Sarker et

al., 2016). Simply, Nile tilapia is able to turn nearly all of the food it consumes into weight, with a large portion of that weight being protein. Second, there is a low cost associated with the production of Nile tilapia. Production of Nile tilapia can be as low as 0.55 US dollars per kilogram of production in tropical countries (Rakocy, 2019). Lastly, high tolerance to stressors allows Nile tilapia to adapt to a range of different environments. Although being a freshwater fish, Nile tilapia can tolerate brackish water, with salinity concentrations of 15 ppt (Jaspe & Caipang, 2011) or even higher (Malik et al., 2018). Although best growth is achieved at the tropical temperatures Nile tilapia are native to, they can adapt to temperatures from 24ºC - 37ºC (Baras et al., 2001; Workagegn, 2012). Nile tilapia can also tolerate a wide range of stocking densities (Azaza et a., 2013). This high degree of adaptability, in addition to the high feed conversion and low cost of production, make Nile tilapia a desirable fish for mass production.

There are many stressors associated with rearing fish in aquaculture. In these artificial environments, fish are exposed to stressors they would not normally encounter in their natural environment. Poor water chemistry, such as increases in ammonia, acidification, and low oxygen levels in the water (hypoxia), cause stress to the fish (Barton, 2002) (Fig. 3). There are many stressors associated with the production of fish as well. Crowded conditions, increased handling, and transportation of fish all cause stress to the animal (Barton, 2002). Meanwhile, growing concerns from climate change resulting in warmer temperatures, as well as water pollution from human activity, are going to burden the production of these animals (Sarà et al., 2018). All of these stressors have the potential to cause harm to the fish, but there is growing concern about the combined effects of these stressors. The interaction of stressors in aquaculture are going to be a major suppressor of aquaculture growth moving forward (Sarà et al., 2018). Even though Nile tilapia have a high tolerance to stress, it does not mean they are immune to stress. Nile tilapia are still susceptible, albeit to a less degree, but an increase in stress is expected to have many negative repercussions on the fish.

The consequences of stress on fish is great, producing varied responses over time. Stress can be considered either acute or chronic. Acute stress, or short-term stress, is generally seen as adaptive, while chronic stress, or long-term stress, is generally seen as maladaptive (Dhabhar, 2009). The stress response is usually considered beneficial, and in and of itself helps protect the

organism from a perceived threat. Under acute stress, metabolic changes occur to protect the organism from homeostatic disturbances, but chronic stress leads to exhaustion of the organism (Barton and Iwama, 1991). It's clear that stress itself is not the issue, but the duration of stress is of greatest concern (Dhabhar, 2009). When an organism is stressed, it goes through three stages: 1) the alarm stage, 2) the adaptive stage, and 3) the exhaustion stage (Barton & Iwama, 1991). In the alarm stage, rapid physiological responses occur, preparing the organism for flight-or-fight (Barton, 2002). This allows the organism to quickly remove itself from any perceived stress, if possible. If the organism is not able to remove itself from the stressor, it will need to adapt to that stress (Barton & Iwama, 1991). In the adaptive stage, energy is used to maintain homeostasis, allowing the organism to survive in stressful conditions (Barton & Iwama, 1991). At a certain point, the organism may no longer be able to maintain homeostasis, resulting in exhaustion (Barton & Iwama, 1991). With exhaustion, the organism succumbs to stress, resulting in many deleterious consequences, with death being the typical endpoint (Barton & Iwama, 1991).

Stress in the aquaculture environment has many different physiological consequences (Fig. 3). Primary responses to stress are focused on neuroendocrine changes within the organism (Barton, 2002). Primary responses to stress are associated with the production of catecholamines, epinephrine and norepinephrine, after a perceived threat (Barton, 2002). Production of catecholamines stimulates the hypothalamic-pituitary-interrenal (HPI) axis in fish, which is modulated through the production of corticotrophin-releasing factor (CRF) in the hypothalamus, stimulating release of adrenocorticotrophin (ACTH) from the anterior pituitary (Barton, 2002). Stimulation of the HPI axis results in the secretion of corticosteroids, such as cortisol, from interrenal cells, releasing the corticosteroids into the circulatory system (Barton, 2002). Secondary responses to stress are generally focused on the many metabolic changes caused by the release of corticosteroids (Barton, 2002). Corticosteroids disrupt osmoregulation, causing variations in the concentration of water, as well as sodium and chloride ions (Barton, 2002). Corticosteroids also produce changes in blood glucose, resulting in greater concentrations (Barton, 2002). Other consequences include the upregulation of heat shock proteins (HSP) to prevent degradation and misfolding of proteins, as well as changes in hematocrit (percentage of red blood cells in the blood) and immune functioning (e.g. lysozyme activity) (Barton, 2002). Lastly, the accumulation of these consequences can produce whole-organism changes (Barton,

2002). Corticosteroid release due to stress can cause compromised growth and immunity in fish (Barton, 2002). Under stress, feeding may decrease and the organisms may become more susceptible to diseases. Ultimately, this may lead to death if the stressor(s) is not removed (Barton, 2002).

As stress leads to compromised growth and immunity, fish farmers need to intervene to prevent loss of the fish population. In China, spread of infection disease has led to losses of aquaculture product amounting to tens of billions of US dollars (Liu et al., 2017). Due to the global market pushing for increases in production, crowding of fish at aquaculture facilities is of growing concern, especially in regards to stress and disease outbreak. This can lead to treating the water with chemicals in the attempt to react to these negative consequences of stress. Antibiotics have been used to help treat bacterial infections in fish and prevent death or the spread of infection (Wang & Lu, 2016), especially in China and other Asian countries (Liu et al., 2017), where global aquaculture production is concentrated. A near majority of all antibiotics used in China are placed into animal feed, even as Western countries have been heavily regulating the use of antibiotics in animal feed (Hvistendahl, 2012, Watts et al., 2017). The prevalence of antibiotics puts a selective pressure on antibiotic resistance. Many different antibiotic resistance genes have been detected in aquaculture (Watts et al., 2017). Antibiotic residues have also been reportedly detected in over 50% of aquacultured fish products from areas in China (Wang et al., 2017), with concentrations of up to 0.1mg per kilogram of fish (Hvistendahl, 2012). Because of this, aquaculture is currently the largest exposure for antibiotics in the human population (Wang et al., 2017). Since consumers are generally unaware of where their fish product is produced (Vanhonacker et al., 2011), many individuals may be exposed to these antibiotics without even noticing. The use of antibiotics also pollutes the environment with antibiotic resistance genes, with fish feces providing a reservoir of these in the environment (Caruso, 2016). These antibiotic resistance genes can also be deposited into sediments, further propagating the spread of resistance (Watts et al., 2017). Antibiotics are not the only concern though. Other chemicals, such as antifoulants, anesthetics, and disinfectants are commonly used as well in aquaculture (Burridge et al., 2010). Antifouling paint containing copper is regularly used to prevent buildup of aquatic microorganisms, plants, and animals on caging (Braithwaite et al., 2006), but this paint is known to leach copper into the water, causing harm to non-target organisms (Singh and

Turner, 2009). Disinfectants are regularly used to inhibit growth of unwanted microorganisms and prevent disease in fish (Burridge at al., 2010). Hypochlorite, chlorine dioxide, and formalin 40% have all been reportedly used in aquaculture production (Burridge et al., 2010). The negative consequences of stress in aquaculture are vast, requiring intervention to not only promote wellbeing of the fish, but to ensure adequate growth for production. Clearly antibiotics and the use of other chemicals are not the solution due to their negative side effects, but they are still prevalent in the food source and further polluting the environment.

Nutraceuticals, which are functional foods or food components with innate health benefits, may be particularly effective in modulating the stress response, thereby boosting immunity and growth. In addition, they may reduce the use of antibiotics in the aquaculture system. Many studies have shown that the addition of nutraceuticals to the diet of fish results in increased growth and immunity. Vitamin C added to fish feed has been shown to increase growth and increase disease resistance against a number of fish pathogens (Roosta et al., 2014). Additionally, the use of clove basil oil and ginger oil in feed have both been shown to increase growth in Nile tilapia (Brum et al., 2017). Ginger oil was shown to be particularly effective in improving immunity, resulting in increased macrophage phagocytosis, upregulation of immunoglobulins, and decreased susceptibility to pathogens (Brum et al., 2017). The approach to using nutraceuticals in the fish diet is to act in a more proactive manner, whereas the current use of chemicals and antibiotics can be considered more of a reactive response to treating the side effects of stress.

Specifically, isoflavones, which are plant hormones most prevalent in the legume family, may be especially effective in this role. Isoflavones can be derived from hundreds of different plants, such as red clover, alfalfa, kudzu, soy, and even grapes (Klejdus et al., 2005). Isoflavones are typically localized within the roots and seeds of these plants. These isoflavones can become activated through animal metabolism (Pilsáková, 2010). Isoflavones (Fig. 4) are structurally similar to estrogen, allowing them to act on estrogen receptors in animals by weak binding (Pilsáková, 2010). By acting through estrogen receptors, isoflavones have the potential to modulate numerous physiological processes, such as inflammation, lipid metabolism, blood pressure, apoptosis, and the hypothalamus-pituitary axis (Pilsáková, 2010). The isoflavone

puerarin has previously been demonstrated to help boost immunity in fish, possibly due to the increase in blood flow (Hossain and Mustafa, 2014). There are many other possible benefits of isoflavone use as well. The isoflavone genistein has shown the potential to increase the feeding efficiency in fish through the increase in protein utilization (Torno et al., 2018), while isoflavones from soybean have been previously shown to have potent antioxidant capabilities (Yue et al., 2010). Due to the phenolic hydroxyl structure associated with certain isoflavones, these isoflavones have also demonstrated inherent antimicrobial properties (Mukne et al., 2011). The presence of the phenolic hydroxyl group allows for binding to microbial enzymes, inhibiting the metabolic pathways used by these organisms (Mukne et al., 2011). There are many possible mechanisms for isoflavones to promote overall health and wellness in the fish.

The objectives of these studies are to determine the effects of isoflavones (pure isoflavone isolates: puerarin and genistein, and crude isoflavone extracts: kudzu, red clover, and soybean) on the modulation of stress, immunity, and growth in Nile tilapia. Stress will be examined temporally to determine the possible benefits of isoflavone supplementation at different stages of stress. Both pure isoflavones and crude isoflavones will be examined to better understand the individual and combined effects of these isoflavones.

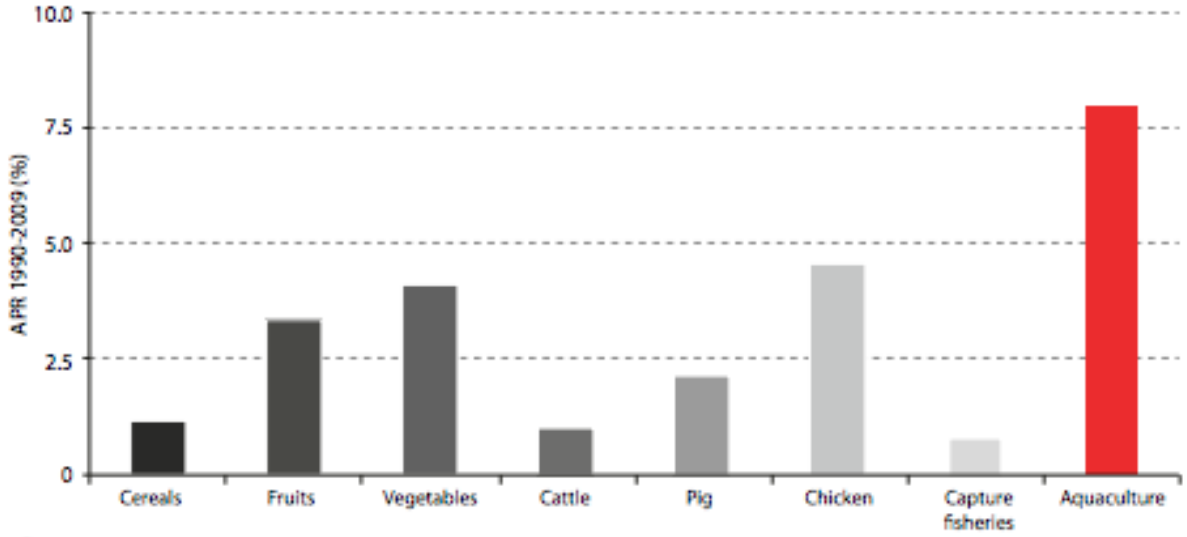

Figure 1: Annual growth rates of aquaculture compared to other food sectors (obtained from Troell et al., 2014)

Figure 2: Yearly aquaculture production (in millions of tonnes) of Nile tilapia (obtained from FAO, 2018)

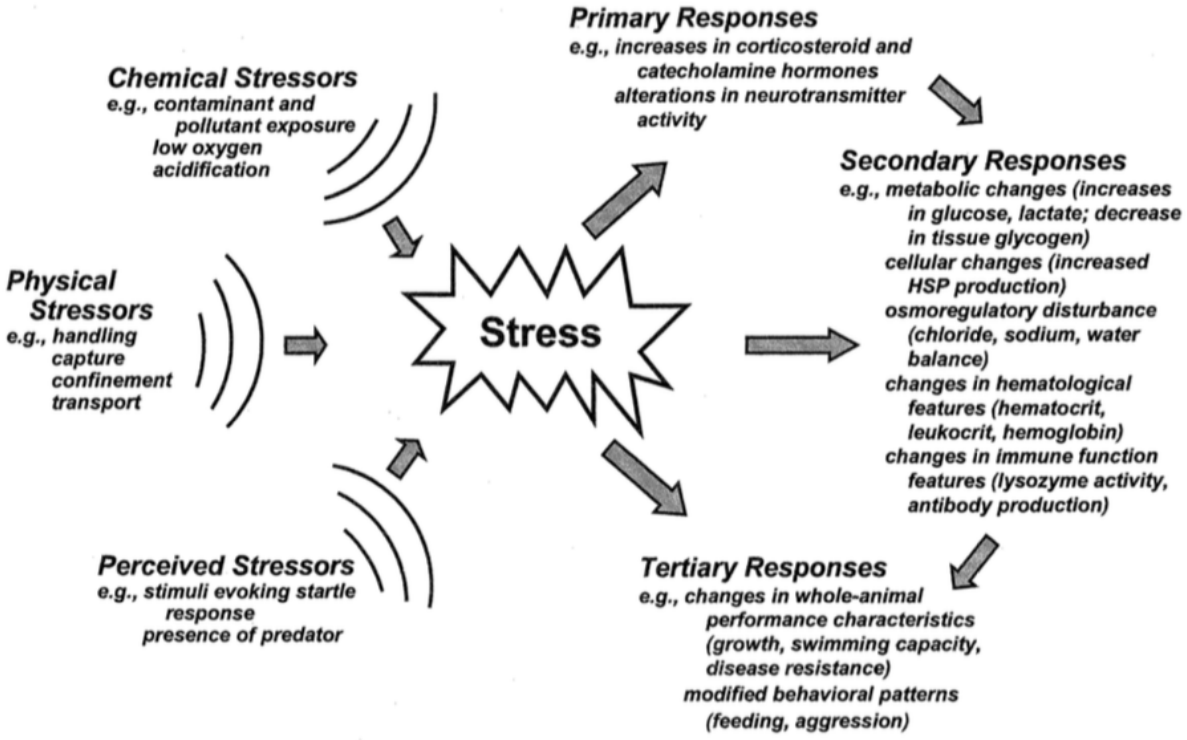

Figure 3: Identification of stressors in the aquaculture environment and the negative consequences of stress on fish (obtained from Barton, 2002)

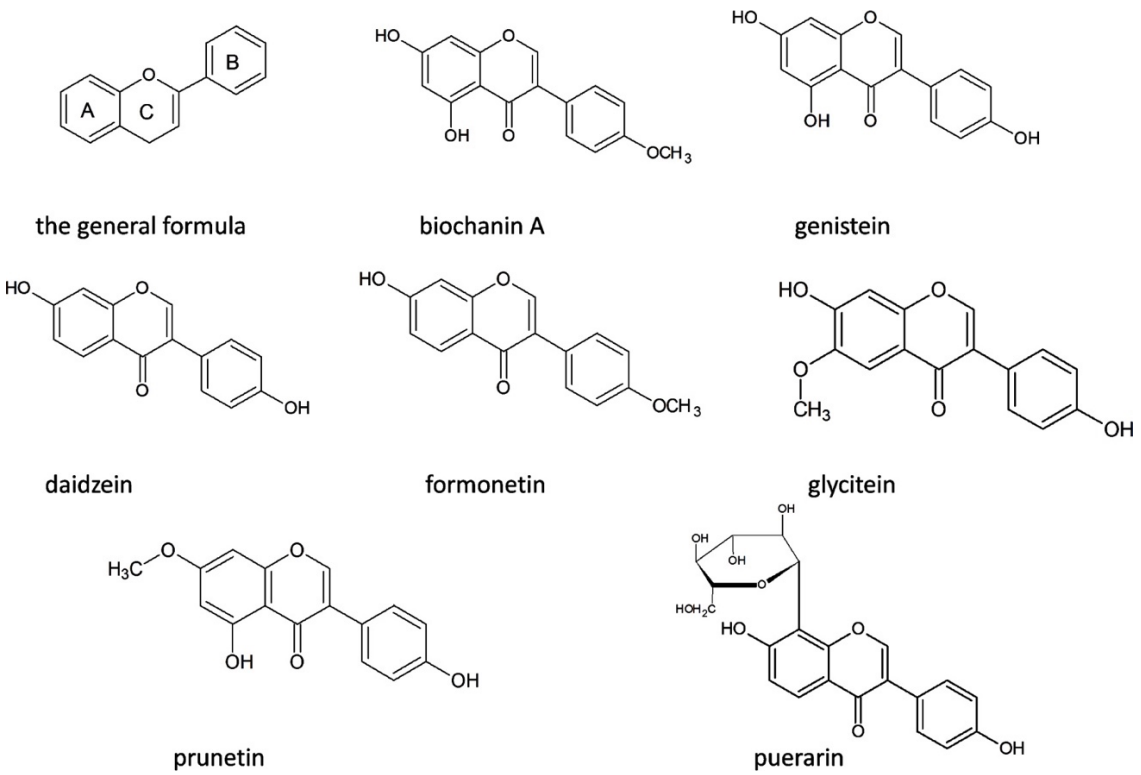

Figure 4: Molecular structure of various isoflavones isolated from the legume family (obtained from Blicharski and Oniszczuk, 2017).

COMPARISON OF PURE ISOFLAVONE ISOLATES ON STRESS

Introduction

Production and consumption of fish has been rapidly growing over the past decades due to the use of aquaculture (Troell et al., 2014). This increase in production has the potential to feed a growing global population. Nile tilapia (*Oreochromis niloticus*) is a particular species of interest, due to their high conversion of feed into product (de Silva, et al., 2017; Sarker et al., 2016), relatively low cost of production (Rakocy, 2019), and their relatively high tolerance to a wide range of growing conditions (Jaspe & Caipang, 2011; Malik et al., 2018; Baras et al., 2001; Workagegn, 2012; Azaza et a., 2013). With this increase in production also comes an increase in stress experienced by the organisms in these artificial environments. Stress in aquaculture is a growing concern, particularly due to the consequences of stress on growth and immunity (Barton, 2002). Stressed fish have been shown to have decreased growth and hindered immunity (Barton, 2002). Unfortunately, chemical antibiotic use is too common of a practice in treating the negative side effects of stress, resulting in the promotion of antibiotic resistance genes and antibiotic residues passed onto consumers through consumption (Watts et al., 2017; Burridge et al., 2010).

The use of nutraceuticals, particularly isoflavones, show the potential to prevent stress, reducing the negative consequences of stress on growth and immunity. Many different isoflavones are produced in the legume family, such as biochanin A, daidzin, genistein, and puerarin, and many others, all which share similar molecular structures (Fig. 4). Collectively, there have been numerous health benefits associated with isoflavone consumption. Ruiz-Larrea et al. (1997) quantified the antioxidant properties of numerous isoflavones by comparing their respective antioxidant properties with vitamin C, a well-known antioxidant. The antioxidant properties of these isoflavones varied greatly (Ruiz-Larrea et al., 1997). Compared to vitamin C, the isoflavone ononin had minimal to no additional antioxidant activity. The isoflavones genistin and daidzein produced a one-fold increase in antioxidant activity compared to vitamin C alone, but genistein produced a three-fold increase in antioxidant activity compared to vitamin C alone. Many of these same isoflavones have also been tested against a range of human cancer cell lines

to determine apoptotic effects of isoflavones (Yanagihara, 1993). Biochanin A and genistein were shown to be particularly effective at promoting apoptosis in a variety of human cancer cell lines, but other isoflavones, such as daidzein and genistin, showed little apoptotic effects. This strongly suggests that although isoflavones may be derived from similar sources and share a similar molecular composition, each individual isoflavone may have its own associated health benefits.

Genistein has been heavily studied, likely due to the importance of soybean as a global food source since genistein is found most abundantly in soybean (Ganai & Farooqi, 2015). This isoflavone is a potential treatment for many diseases. Genistein has been shown to reduce cardiovascular inflammation, reducing the cause of cardiovascular diseases such as atherosclerosis and arthritis (Si & Liu, 2007; Verdrengh et al., 2003). Research is also focusing on the use of genistein to reduce allergies (Ganai & Farooqi, 2015). Genistein is shown to reduce degranulation of mast cells and reduce constriction of the bronchii. It also has been demonstrated to have anthelmintic properties, resulting in paralysis and death of parasitic worms (Tandon et al., 1997). Additionally, genistein has shown to have selective effects on cancer cells (Pool-Zobel, et al., 2000). At appropriate concentrations, genistein demonstrated the ability to promote DNA strand breakage in human colon cancer cell lines, while having no effect on normal colon cells. Researchers are continuing to investigate the wide range of potential health benefits associated with genistein.

Another isoflavone, puerarin, is found in high concentration in kudzu (*Pueraria*) species (Jiang et al., 2005). Comparatively, less research has been focused on the health properties of puerarin, possibly due to the decreased occurrence of this isoflavones in human diets. Even so, research suggests there are diverse health benefits associated with puerarin, such as increased blood flow and promotion of immunity (Hossain and Mustafa, 2014). Focusing on *Staphylococcus aureus* infection in alveolar cells, puerarin was not found to directly inhibit bacterial growth (Tang et al., 2014). Instead, puerarin was able to downregulate the production and secretion of alpha-hemolysin. Alpha-hemolysin creates pores in alveolar cells, ultimately causing cell death (Tang et al., 2014). By downregulating alpha-hemolysin, puerarin was able to protect alveolar cells against cytotoxicity (Tang et al., 2014). Puerarin also has neuro-protective benefits, as shown by

an overall reduction in infarct volume after a stroke (Wu et al., 2007). Due to the wide range of health benefits from genistein and puerarin, as well as other isoflavones, these may be possible candidates as nutraceuticals to minimize the damage of stress on fish.

The objective of this experiment was to determine the effects of pure isoflavone isolates, genistein and puerarin, on the effects of stress in Nile tilapia. Genistein and puerarin were hypothesized to reduce stress parameters, thereby showing improved growth and immunity parameters (Table 1).

Methods

Fish Acquisition and Maintenance

Fingerling Nile tilapia (*Oreochromis* niloticus) were obtained from Troyer Aqua Farm (Indiana, USA) and reared in a recirculating system in the Life Science Resource Center on the Purdue University Fort Wayne campus. Five hundred Nile tilapia were randomly distributed into one of four tanks (n = 125 per tank) across two systems, with each tank being fed its own unique diet (Fig. 5). Twenty percent water changes were performed on a weekly basis for the duration of the experiment, with water chemistry being measured on a weekly basis. Water chemistry was maintained at a healthy level (ammonia < 0.25 ppm, nitrite < 0.5 ppm, nitrate < 40 ppm, dissolved oxygen > 7.0 mg/L). The pH and temperature ranged from 7.2-7.6 and 25-28°C, respectively. A photoperiod of 12 hours daylight and 12 hours night was maintained for the duration of the experiment.

Table 1: Hypothesized results of stress on various physiological and growth parameters. Supplementation of isoflavones for stressed fish is expected to improve these parameters.

Parameter	Stress	Isoflavone Supplementation
Serum Cortisol	↓	↑
Blood Glucose	↑	↓
Hematocrit	↑	↓
Plasma Protein	↑	↓
Hepatosomatic Index	↓	↑
Spleen-Somatic Index	↓	↑
Length	-	-
Weight	↓	↑
Biomass	↓	↑
Specific Growth Rate	↓	↑
Feed Intake	↓	↑
Feed Conversion Ratio	↑	↓
Protein Efficiency Ratio	↓	↑
Condition Factor	↓	↑

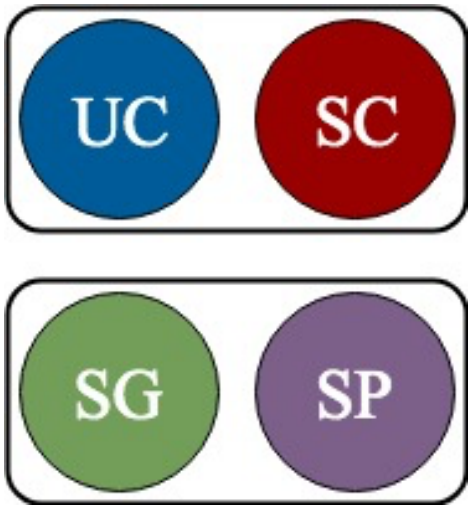

Figure 5: Tank design with diets labeled. UC = unstressed fish fed commercial feed, SC = stressed fish fed commercial feed, SG = stressed fish fed genistein-supplemented feed, SP = stressed fish fed puerarin-supplemented feed.

Diets

AquaMax® Fingerling Starter 300 commercial feed was obtained from Purina® (Missouri, USA), with nutritional information provided in Table 1. Fish were fed once daily at 2% of mean body weight, with fish being weighed on a weekly basis to determine mean body weight. Hydrocortisone was used to induce stress in fish, which has been reported previously (Barton, et al, 1987). To stress fish, 100 mg of hydrocortisone, 98% (Acros Organics, New Jersey, USA) dissolved in pure ethanol was mixed with one kg of commercial feed in an enclosed container. Feed pellets were spread out on a tray and allowed to air dry overnight before use. The pure isoflavones genistein (LC Laboratories, Massachusetts, USA) and puerarin (Alfa Aesar, Massachusetts, USA) were added to the feed in a similar fashion as hydrocortisone, with the exception that 2 g of respective isoflavone was added per kg of commercial feed. This concentration of isoflavone has previously demonstrated positive effects when used in diet supplementation of stressed Nile tilapia (Hossain et al., 2013; Hossain and Mustafa, 2014). Feed was stored in a sealed container at 4ºC.

Experimental Design

Fish were allowed to acclimate for two weeks prior to starting the experiment, during which time they were fed commercial feed until satiation. Fish were fed one of the four experimental diets: 1) unstressed fish fed commercial feed (UC), which served as a negative control for stress, 2) stressed fish fed commercial feed (SC), which served as a positive control for stress, 3) stressed fish fed genistein supplemented feed (SG), and 4) stressed fed fish puerarin-supplemented feed (SP) (Fig. 4). Fish were starved for 24 hours before sampling. Six Nile tilapia were randomly sampled from each of the four experimental diets at day 0, before use of the experimental diets, to serve as a single baseline for all groups for the growth portion of this study. Nile tilapia ($n = 6$) were randomly sampled from each of the four tanks on days 3, day 21, and day 49 after initiation of the experimental diets, with day 49 serving as the terminal sampling for both the physiological and growth portions of this study.

Fish were euthanized by adding 200 mg of Tricaine-S (MS-222) (Western Chemical, Washington, USA) to one liter of water, waiting until operculum movement and response to

stimuli ceased. Weight and length were collected at every sampling period. At day 3, day 21, and day 49, blood was collected from the caudal vein of each fish using a heparinized (Sequester-Sol, Florida, USA) 25-gauge needle (BD Syringe, New Jersey, USA). To obtain plasma, blood was immediately centrifuged at 10,000 rpm for 5 minutes before clotting occurred. To obtain serum, blood was allowed to sit on ice for one hour so clotting could occur. After clotting, the blood was spun at 5000 rpm for 10 minutes using a centrifuge. Excess serum was stored at -80ºC until ready for use. Fish were then dissected to collect the liver and spleen.

Physiological Parameter

Serum cortisol concentration was analyzed using a commercial cortisol enzyme-linked immunosorbent assay (ELISA) kit purchased from Cayman Chemical (Michigan, USA). Procedure was followed according to manufacturer's guidelines (Cayman, 2017). Briefly, serum was removed from -80ºC and allowed to thaw before use. First, ELISA buffer was added to a 96-well plate, followed by the standard cortisol concentrations or serum sample. Next, cortisol acetylcholinesterase (AChE) inhibitor was added before finally adding cortisol ELISA monoclonal antibodies. Plates were covered in plastic film and allowed to incubate overnight at 4ºC. After incubation, plates were emptied and rinsed five times using wash buffer. Ellmen's reagent was added to each well, plates were covered with plastic film, and incubated at room temperature for 90 minutes using an orbital shaker. Plates were read at 420nm using a Multiskan GO (Thermo Scientific, Massachussets, USA) plate reader. Data was analyzed using a four-parameter logistic fit with log concentration of cortisol plotted on the x-axis and $\%B/B_0$ plotted on the y-axis.

Blood glucose concentration was determined using heparinized blood. One drop of blood from each sample was added to a FreeStyle Lite (Abbott, California, USA) blood glucose test strip and read using a FreeStyle Lite (Abbott, California, USA) glucometer. The FreeStyle Lite glucometer has shown to be one of the most accurate glucometers on the market, with a 96% compliance rate (Klonoff et al., 2018).

To determine hematocrit (packed cell volume), heparinized blood was added to a capillary tube, sealed at one end using a critocap, and spun at 10,000 rpm for five minutes using a

microcentrifuge. The capillary tube with separated blood was placed on a microhematocrit capillary tube reader and the percentage of red blood cells was read.

Plasma protein concentration was determined using a VEE GEE CLX 1 (Washington, USA) refractometer. 2 drops of plasma were added to the surface of the prism. The plate lid was closed and placed under a bright light to view the blue-white boundary, indicative of the plasma protein concentration. The refractometer was properly cleaned between samples.

To determine the organ-somatic indices, both the spleen and liver were weighed after dissection. Hepatosomatic index (HSI) and spleen-somatic index (SSI) were calculated by the following equation:

$$\text{HSI: } \frac{\text{Liver weight (g)}}{\text{Total body weight (g)}} * 100 \qquad \text{SSI: } \frac{\text{Spleen weight (g)}}{\text{Total body weight (g)}} * 100$$

Growth Parameters

Initial length (L_I) in cm and initial weight (W_I) in g were collected at day 0. Initial biomass (B_I) in g was calculated by the addition W_I from the six-fish sample. Using different fish by random sampling, final length (L_F) in cm and final weight (W_F) in g were collected at day 49. Final biomass (B_F) in g was calculated by the addition W_F from the six-fish sample. Condition factor was calculated as followed: [weight/length^3]. Total feed intake (FI) in g was calculated by the addition of feed added to the tank from each of the six weeks of sampling. Protein intake (PI) in g was calculated by multiplying FI by the proportion of protein in the feed. All growth parameters were calculated according to Table 3.

Statistics

All physiological parameters and the growth parameters length, weight, and FCR were analyzed using a two-way (4x3) multivariate analysis of variance (MANOVA) using diets and time as the independent variables ($\alpha = 0.05$). The two-way MANOVA was followed-up using a series of univariate two-way ANOVAs for dependent variables that had a statistically significant

interaction from the MANOVA, examining diets at every level of time. Dunnett's test (many-to-one comparison) was used for all post-hoc analysis, with SC serving as the diet with which the other diets were compared. Comparing all other diets to only SC was utilized for two reasons: 1) SC is the only diet that has one degree of commonality with all other diets regarding stress and isoflavone supplementation, and 2) to minimize the type 1 (family-wise) error rate due to fewer overall comparisons than testing all possible differences between diets. If there was not a significant interaction for the dependent variable, only the statistically significant main effect of diets was examined using a univariate one-way ANOVA followed by Dunnett's test. Figure 6 provides a graphical summary of the statistical analyses used.

Table 2: Nutritional composition for experimental diets (expressed as % of total feed). UC = unstressed fish fed commercial feed, SC = stressed fish fed commercial feed, SG = stressed fish fed genistein-supplemented feed, SP = stressed fish fed puerarin-supplemented feed.

Diets	**UC**	**SC**	**SG**	**SP**
Crude Protein (%)	50.00	50.00	50.00	50.00
Crude Fat (%)	16.00	16.00	16.00	16.00
Crude Fiber (%)	3.00	3.00	3.00	3.00
Ash (%)	12.00	12.00	12.00	12.00
Calcium (%)	2.55	2.55	2.55	2.55
Phosphorus (%)	1.30	1.30	1.30	1.30
Sodium (%)	0.60	0.60	0.60	0.60
Hydrocortisone (%)	-	0.01	0.01	0.01
Puerarin (%)	-	-	0.20	-
Genistein (%)	-	-	-	0.20

Table 3: Equations used to determine growth parameters

Growth Parameter	**Equation**
Total biomass change (B_Δ)	$B_F - B_I$
Daily B_Δ	$\frac{B\Delta}{\text{Days}}$
Total weight gain (W_Δ)	$W_F - W_I$
Daily W_Δ	$\frac{W\Delta}{\text{Days}}$
Specific Growth Rate (SGR)	$\frac{\ln(W_F) - \ln(W_I)}{\text{Days}} * 100$
Daily FI	$\frac{\text{Total FI}}{\text{Days}}$
Feed Conversion Ratio (FCR)	$\frac{(\text{FI/Fish in Sample})}{\text{Total } W_\Delta}$
Protein Efficiency Ratio (PER)	$\frac{\text{Total } W_\Delta}{(\text{PI/Fish in Sample})}$
Condition Factor (K)	$\frac{\text{Weight}}{\text{Length}^3}$

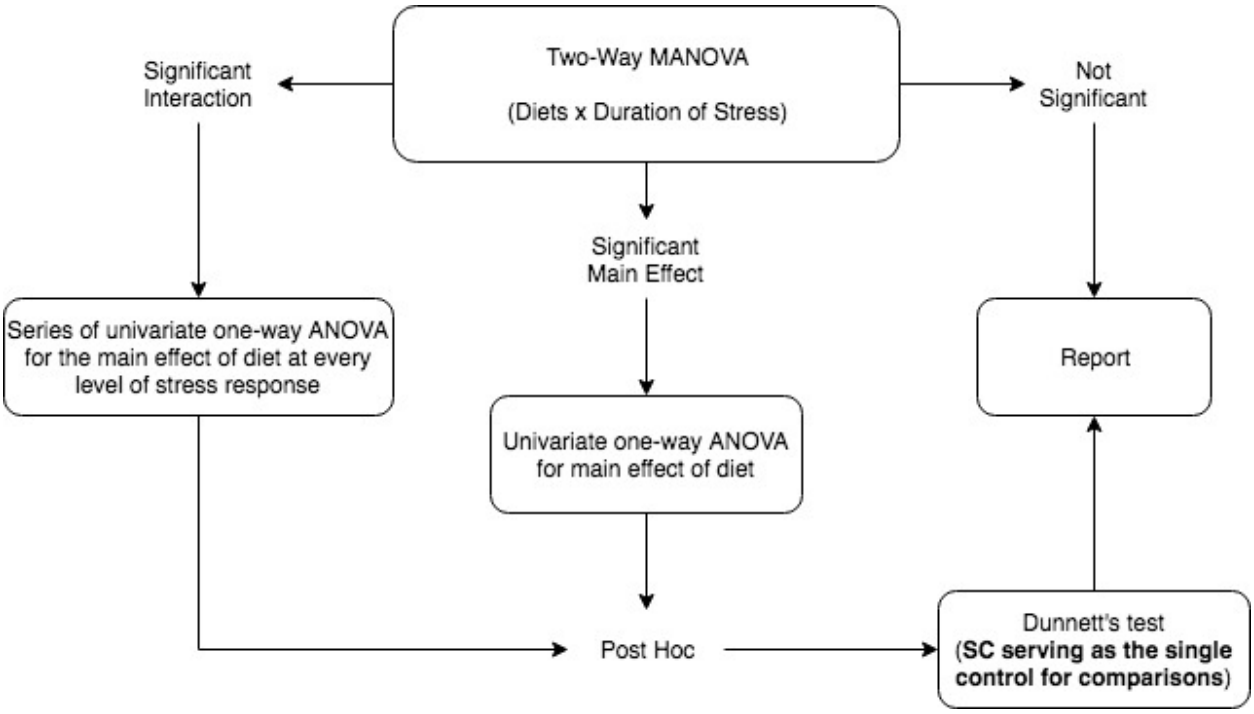

Figure 6: Flow chart for statistical analysis of diets and time on physiological and growth parameters

Results

The results of the MANOVA analysis are presented in Table 4. Focusing on the main effect of diets on cortisol (Fig. 7), all stressed groups showed a decrease in serum cortisol levels. Serum cortisol for UC was 3676 ± 1161 pg/mL, which was significantly greater than SC with 274 ± 56 pg/mL of cortisol in the serum ($p = .001$). Although isoflavone supplemented diets showed overall greater serum cortisol concentration, there were not any significant differences between SC and SG ($p = .954$) or SC and SP ($p = .260$).

Since there was a significant interaction between diets and time for blood glucose ($p = .008$), hematocrit ($p = .037$), and plasma protein ($p > .001$), diets were compared at every level of time (i.e. day 3, day 21, and day 49) (Fig. 8, Fig. 9, and Fig. 10, respectively). At day 3, there was a significant effect of diets on blood glucose concentration ($F(3,20) = 3.703$, $p = .029$). SG had a blood glucose level of 63.5 ± 7.4 mg/dL, significantly greater than SC, which had a blood glucose level of 43.7 ± 1.6 mg/dL ($p = .021$). UC and SP did not significantly differ from SC ($p = .589$ and $p = .999$, respectively). There was also a significant effect of diets on blood glucose concentration at day 21 ($F(3,20) = 6.472$, $p = .003$). At day 21, all stressed groups had a lower blood glucose concentration compared to UC. SC had a significantly lower blood glucose level at 32.2 ± 1.9 mg/dL compared to UC at 42.3 ± 4.2 mg/dL ($p = .035$), but did not significantly differ from SG ($p = .473$) or SP ($p = .677$). Blood glucose levels were comparable between all diets at day 49 ($F(3,20) = .491$, $p = .693$).

Similar to blood glucose, there was a significant effect of diets on hematocrit at days 3 ($F(3,20) = 3.223$, $p = .045$) and day 21 ($F(3,20) = 8.529$, $p = .001$), but not at day 49 ($F(3,20) = .828$, $p = .494$). At day 3, SG had significantly decreased hematocrit to 34.5 ± 1.3 % compared to SC, with a hematocrit of 43.3 ± 3.0 % ($p = .015$), although UC ($p = .200$) and SP ($p = .244$) did not significantly differ from SC. Diets had a significant effect on hematocrit at day 21 ($F(3,20) = 8.529$, $p = .001$), with all stressed groups having elevated hematocrit at day 21 compared to control. While SG and SP did not significantly differ from SC ($p = .148$ and $p = .680$, respectively), SC (50.2 ± 2.0 %) had a significantly increased hematocrit compared to UC (37.3 ± 1.5 %) at day 21 ($p < .001$).

Diets had a significant effect on plasma protein at day 3 ($F(3,20) = 5.053$, $p = .009$), day 21 ($F(3,20) = 14.633$, $p > .001$), and day 49 ($F(3,20) = 9.348$, $p < .001$), although at day 3 and day 49, there were no significant difference between UC and SC ($p = .495$ and $p = .059$, respectively), SC and SG ($p = .166$ and $p = .093$, respectively), and SC and SP ($p = .235$ and $p = .121$, respectively). At day 21, UC had a plasma protein concentration of 4.7 ± 0.4 g/.1L, while all stressed groups showed elevated plasma protein concentration. SC, with a plasma protein concentration of 6.3 ± 0.2 g/.1L, was significantly greater than UC ($p > .001$), but did not significantly differ from SG ($p = .442$) or SP ($p = .506$).

Hepatosomatic index (Fig. 11) was found to not be significantly affected by diets ($p = .517$), but spleen-somatic index (Fig. 12) was significantly ($p = .029$). For spleen-somatic index, SC showed an overall decrease (0.130 ± .007 %) while SP showed an overall increase (0.166 ± .008 %). SC and SP were significantly different from one another ($p = .008$).

Diets did not have a significant effect on length at day 3 ($F(3,20) = 1.406$, $p = .270$) or day 21 ($F(3,20) = .189$, $p = .903$), but did have a significant effect at day 49 *(*$F(3,20) = 5.315$, $p = .007$). At week 7 (Fig. 13), UC had a mean length of 21.4 ± 0.4 cm, while SC had a mean length of 19.8 ± 0.3 cm. SC had a significantly smaller length compared to UC ($p = .005$), but was not significantly different from SG ($p = .969$) or SP ($p = .272$). The same trend was found for weight. There was not a significant effect of diets on weight at day 3 ($F(3,20) = 2.068$, $p = .137$) or day 21 ($F(3,20) = .747$, $p = .537$), but there was a significant effect on weight at day 49 ($F(3,20) = 12.853$, $p < .001$) (Fig. 14). At day 49, SC, with a mean weight of 160.2 ± 5.0 g, which was significantly lower than UC, with a mean weight of 225.0 ± 11.5 g ($p < .001$). While SG and SP had a higher weight than SC, there were not significant differences detected ($p = .356$ and $p = .218$, respectively). Using length and weight to determine condition factor, there was a significant effect of diets on condition factor at day 3 ($F(3,20) = 4.863$, $p = .011$) and day 49 ($F(3,20) = 3.387$, $p = .038$) (Fig. 15), but not at day 21 ($F(3,20) = 1.344$, $p = .288$). Condition factor for SC was 2.20 ± 0.05 at day 3 and 2.08 ± .04 at day 49, while UC reported 2.01 ± .04 at day 3 and 2.29 ± .07 at day 49 for condition factor. At day 3, SC had a significantly greater condition factor compared to both UC ($p = .014$) and SG ($p = .009$), with SG having a condition factor of 1.99 ± .03, but SC had a significantly lower condition factor than UC at day 49 ($p =$

.048). SC did not significantly differ from SG at day 49 (p = .186), and did not significantly differ from SP at day 3 (p = .343) or day 49 (p = 1.000).

Focusing on the qualitative growth data presented in Table 5, UC showed a higher daily and total gain in both B_{Δ} and W_{Δ}, as well as a higher SGR, compared to all stress groups, although this finding did not have replicates. For those stress groups that received IF supplementation, they appeared to have a greater SGR, and daily and total B_{Δ}, and daily and total W_{Δ} when compared to SC. The feed input was greatest for UC, showing more food was required to achieve such growth compared to the stress groups. SG and SP had similar feed inputs as SC, but achieved more growth while having the same feed input. This is represented through FCR and PER, which shows a greater FCR and PER for SG and SP compared to SC. Collectively, UC showed more favorable values across all growth parameters compared to all of the stress diets. With the addition of isoflavone supplementation in the feed, SG and SP showed more favorable values across all growth parameters compared to SC.

Table 4: MANOVA results for the interactive (Int.) effects of time and diet or the main effect (ME) of diet on the combined physiological and growth parameters.

Statistical Test	**Wilks Λ**	***F***	***p***
Overall MANOVA	1.22	(48, 265) = 3.090	<.001
Serum Cortisol (Int.)	-	(6,60) = 1.745	.141
Serum Cortisol (ME)		(3,60) = 7.505	.001
Blood Glucose (Int.)	-	(6,60) = 3.250	.008
Hematocrit (Int.)	-	(6,60) = 2.418	.037
Plasma Protein (Int.)	-	(6,60) = 7.596	<.001
Hepatosomatic Index (Int.)	-	(6,60) = 0.729	.628
Hepatosomatic Index (ME)	-	(3,60) = 0.768	.517
Spleen-Somatic Index (Int.)	-	(6,60) = 1.118	.363
Spleen-Somatic Index (ME)	-	(3,60) = 3.222	.029
Length (Int.)	-	(6,60) = 2.269	.049
Weight (Int.)	-	(6,60) = 4.378	.001
Condition Factor (Int.)	-	(6,60) = 3.871	.002

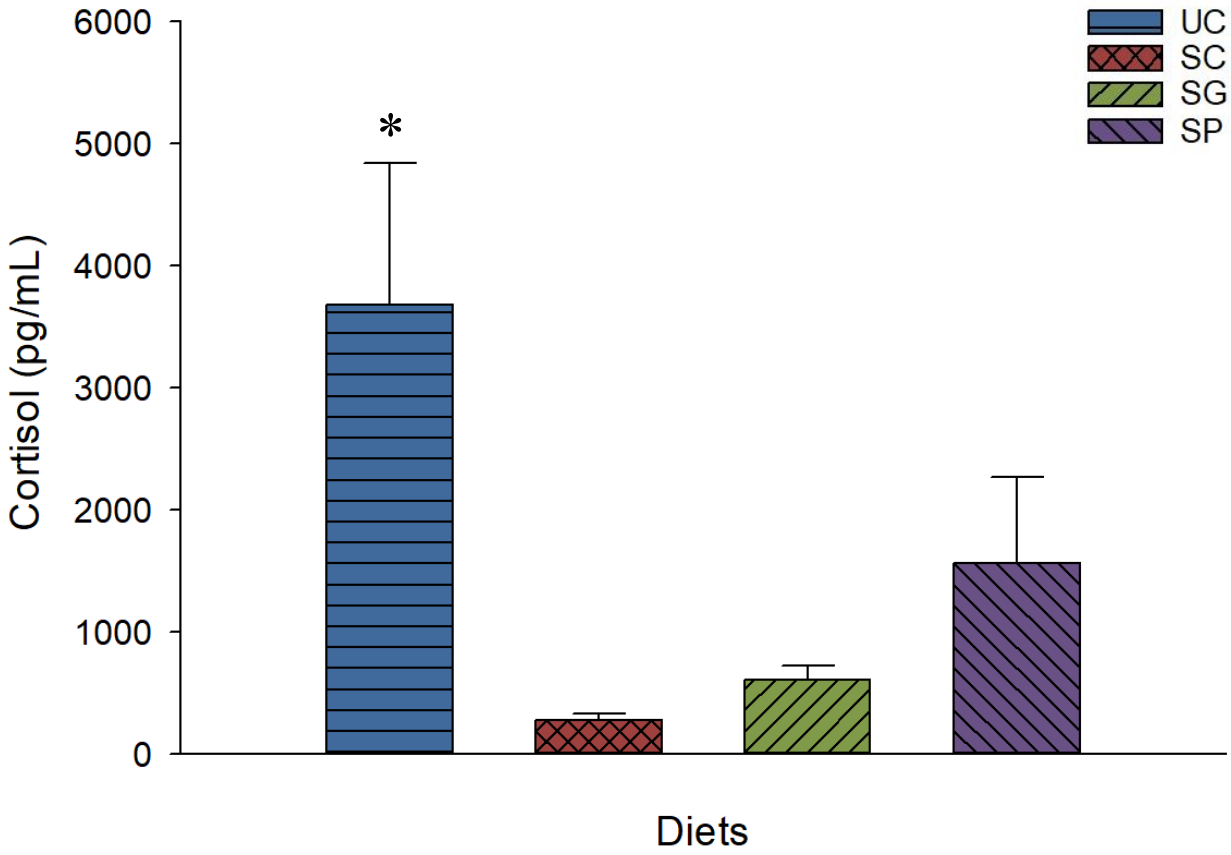

Figure 7: Overall main effect of diets on serum cortisol concentration. UC = unstressed fish fed commercial feed, SC = stressed fish fed commercial feed, SG = stressed fish fed genistein-supplemented feed, SP = stressed fish fed puerarin-supplemented feed. Bars represent mean + SEM; * = $p < .05$ compared to SC.

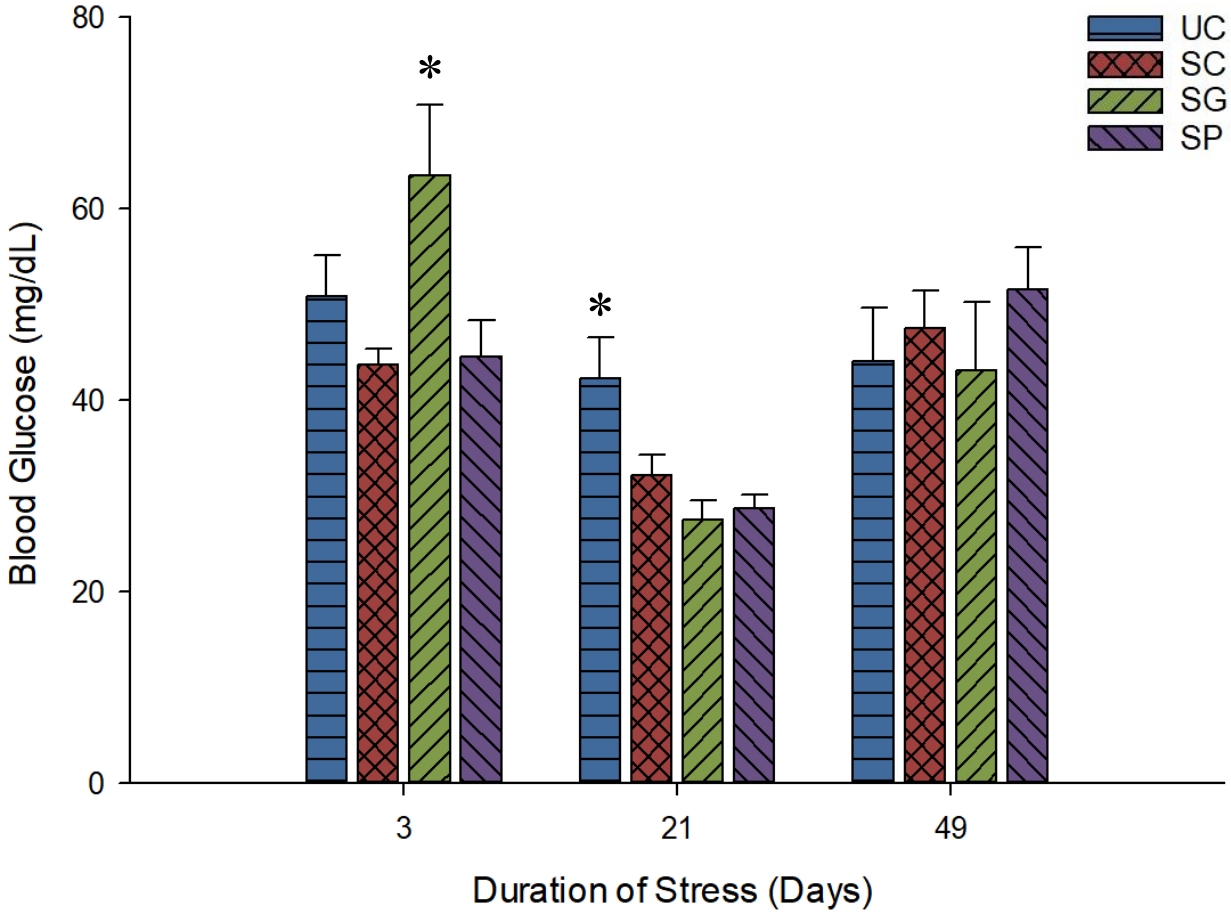

Figure 8: Effect of diets on blood glucose concentration at day 3, day 21, and day 49 post-induction of stress. UC = unstressed fish fed commercial feed, SC = stressed fish fed commercial feed, SG = stressed fish fed genistein-supplemented feed, SP = stressed fish fed puerarin-supplemented feed. Bars represent mean + SEM; * = $p < .05$ compared to SC within each sampling period (days).

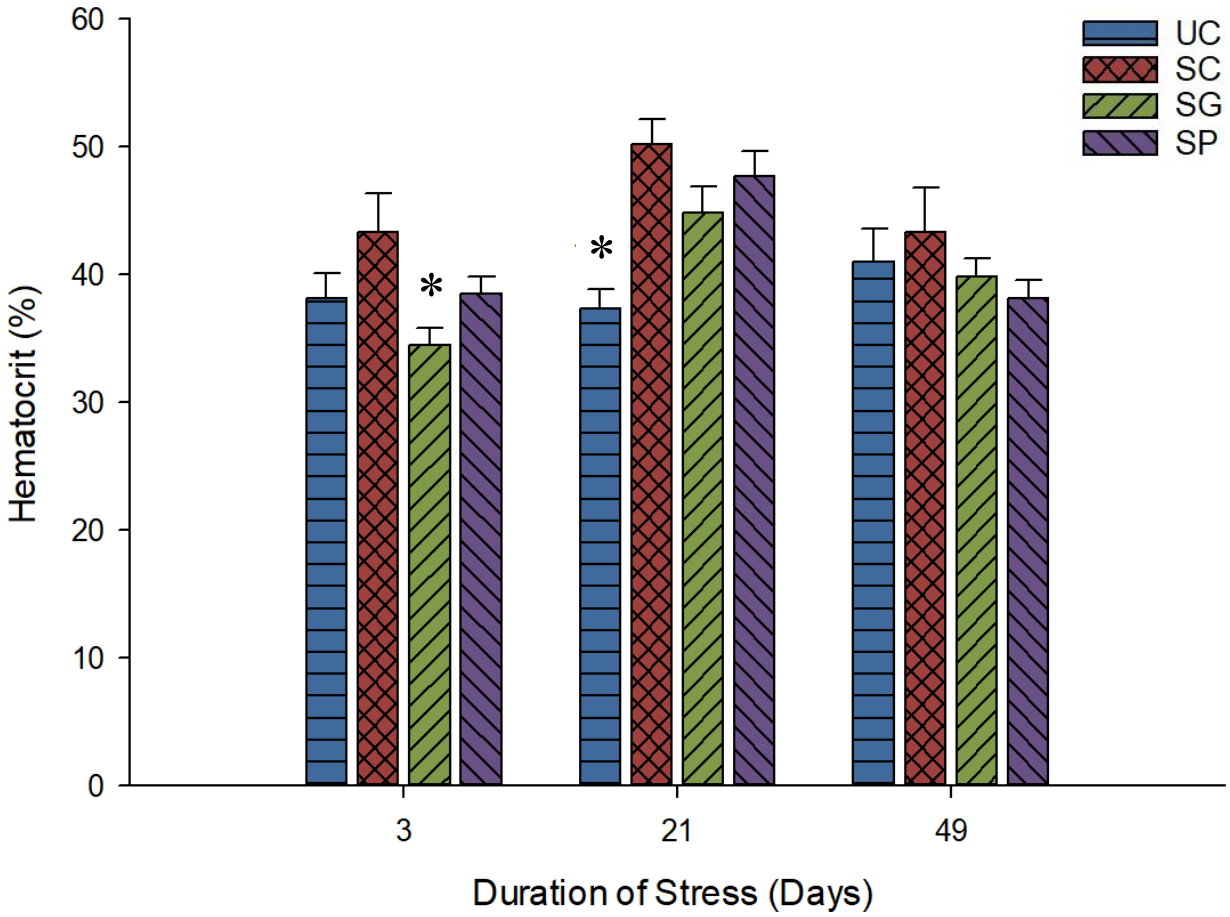

Figure 9: Effect of diets on hematocrit at day 3, day 21, and day 49 post-induction of stress. UC = unstressed fish fed commercial feed, SC = stressed fed commercial feed, SG = stressed fish fed genistein-supplemented feed, SP = stressed fish fed puerarin-supplemented feed. Bars represent mean + SEM; * = $p < .05$ compared to SC within each sampling period (days).

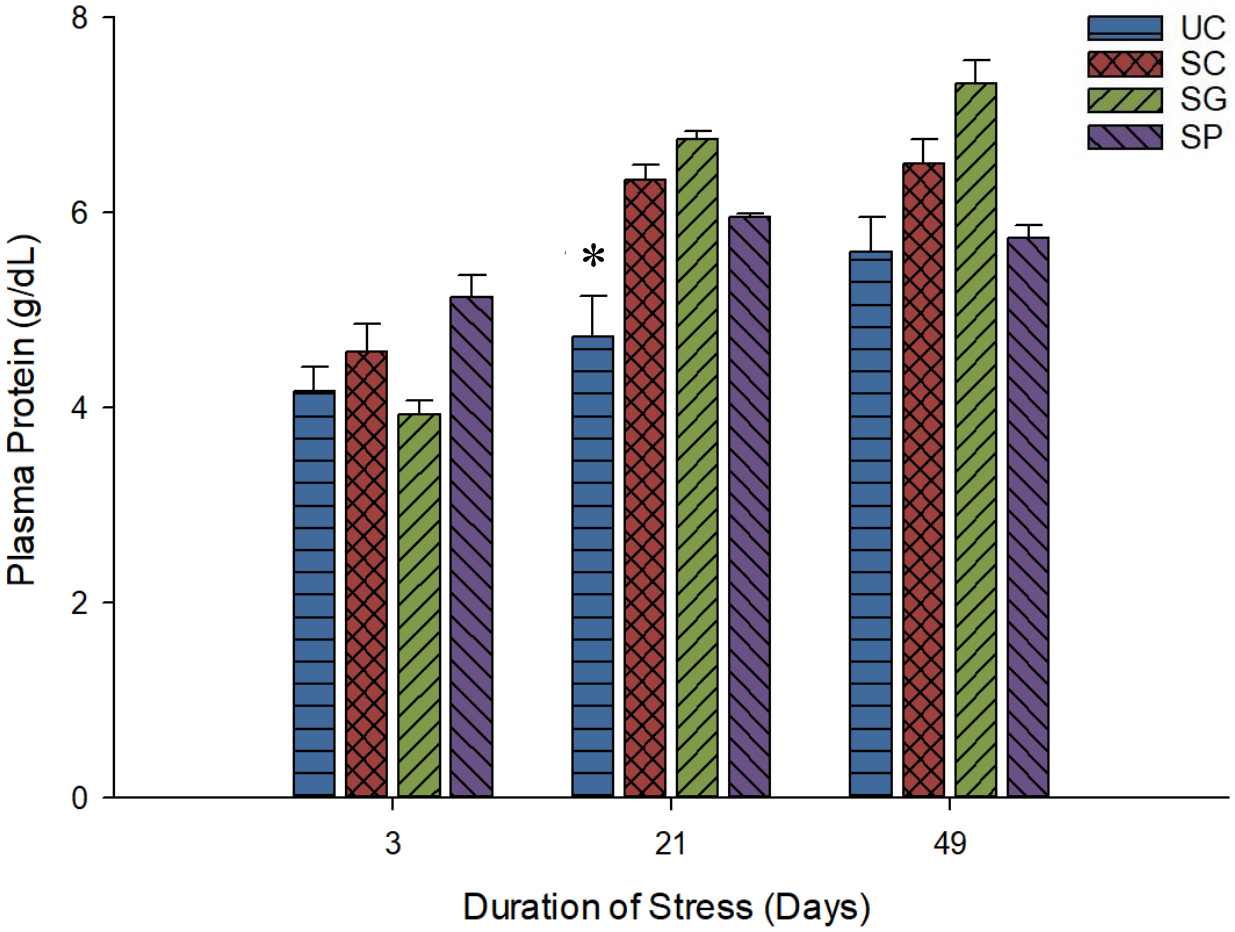

Figure 10: Effect of diets on plasma protein concentration at day 3, day 21, and day 49 post-induction of stress. UC = unstressed fish fed commercial feed, SC = stressed fed commercial feed, SG = stressed fish fed genistein-supplemented feed, SP = stressed fish fed puerarin-supplemented feed. Bars represent mean + SEM; * = $p < .05$ compared to SC within each sampling period (days).

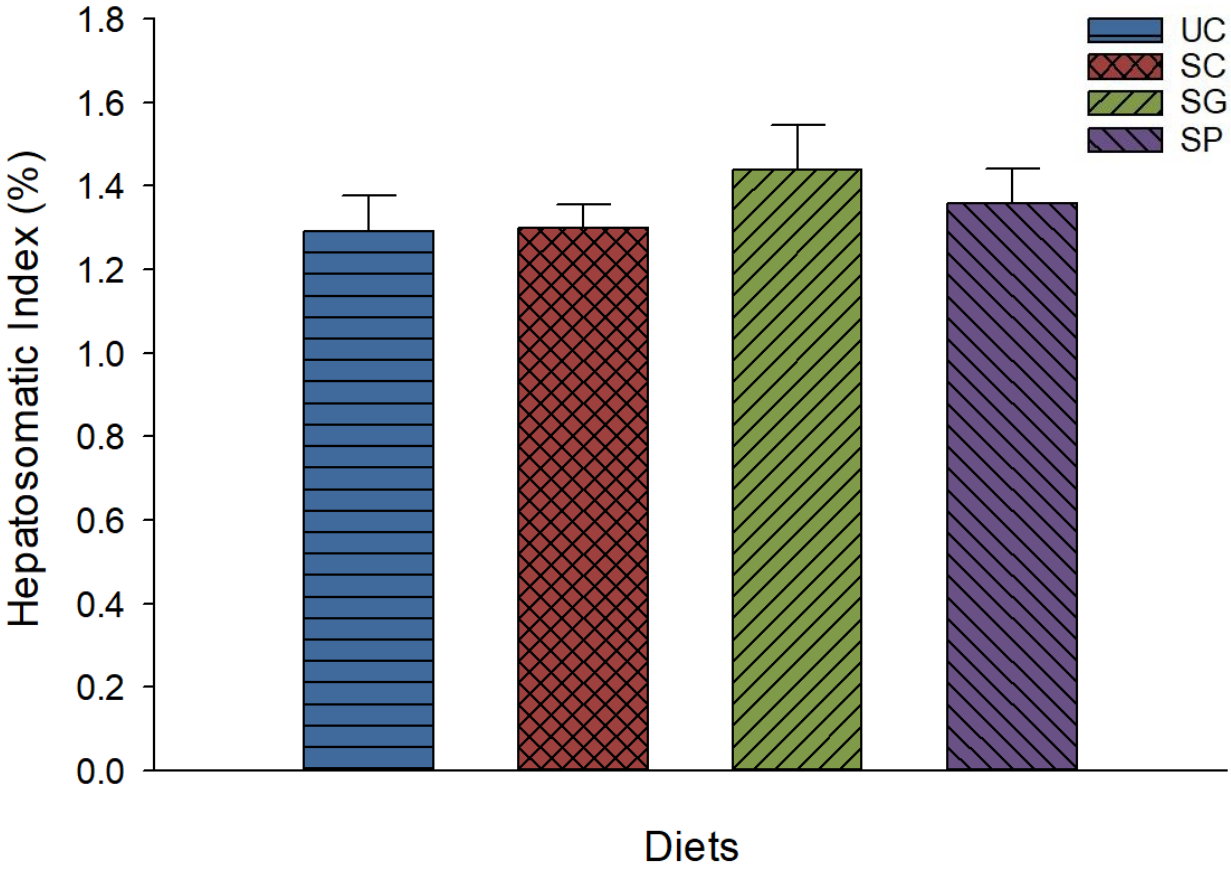

Figure 11: Overall main effect of diets on hepatosomatic index. UC = unstressed fish fed commercial feed, SC = stressed fish fed commercial feed, SG = stressed fish fed genistein-supplemented feed, SP = stressed fish fed puerarin-supplemented feed.
Bars represent mean + SEM.

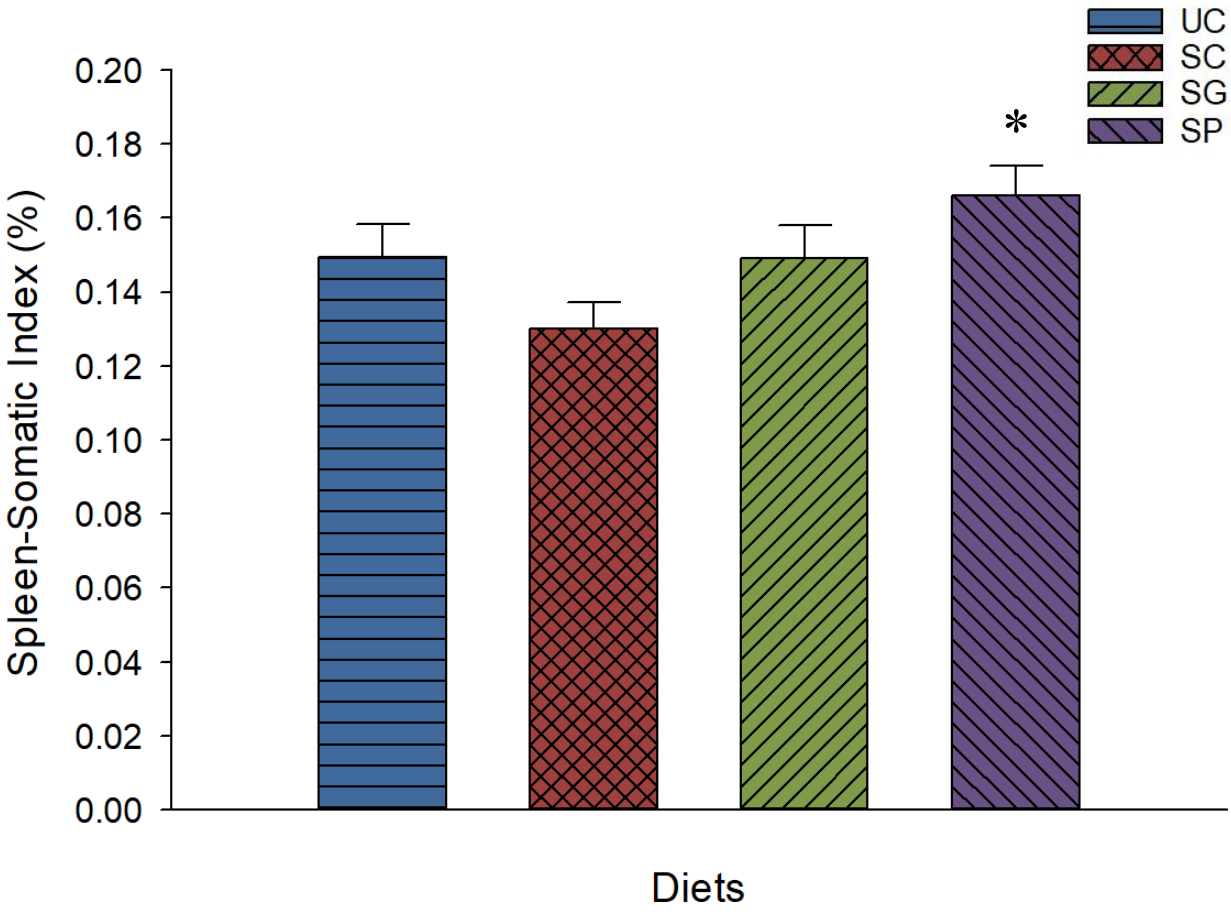

Figure 12: Overall main effect of diets of spleen-somatic index. UC = unstressed fish fed commercial feed, SC = stressed fish fed commercial feed, SG = stressed fish fed genistein-supplemented feed, SP = stressed fish fed puerarin-supplemented feed.
Bars represent mean + SEM; * = $p < .05$ compared to SC.

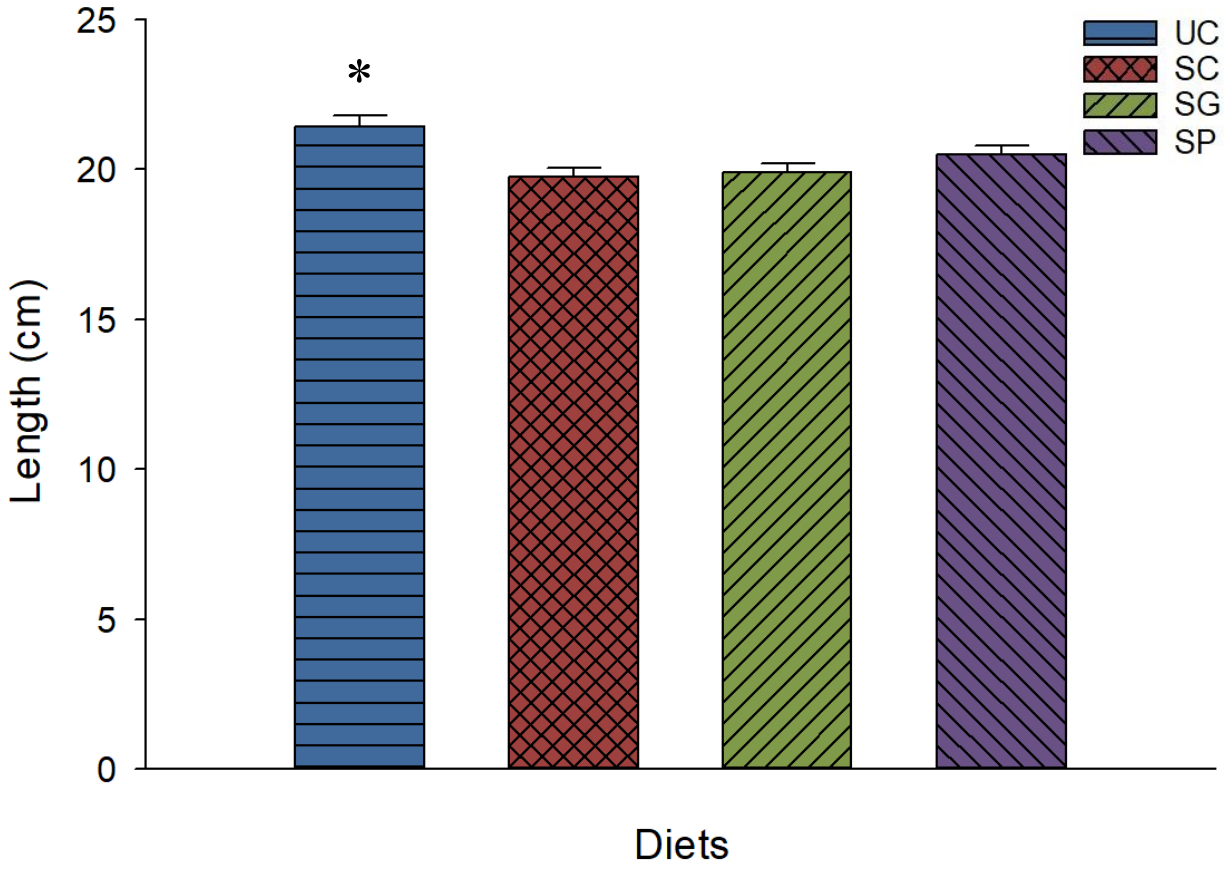

Figure 13: Effect of diets on terminal (day 49) length. UC = unstressed fish fed commercial feed, SC = stressed fish fed commercial feed, SG = stressed fish fed genistein-supplemented feed, SP = stressed fish fed puerarin-supplemented feed. Bars represent mean + SEM; * = $p < .05$ compared to SC at day 49.

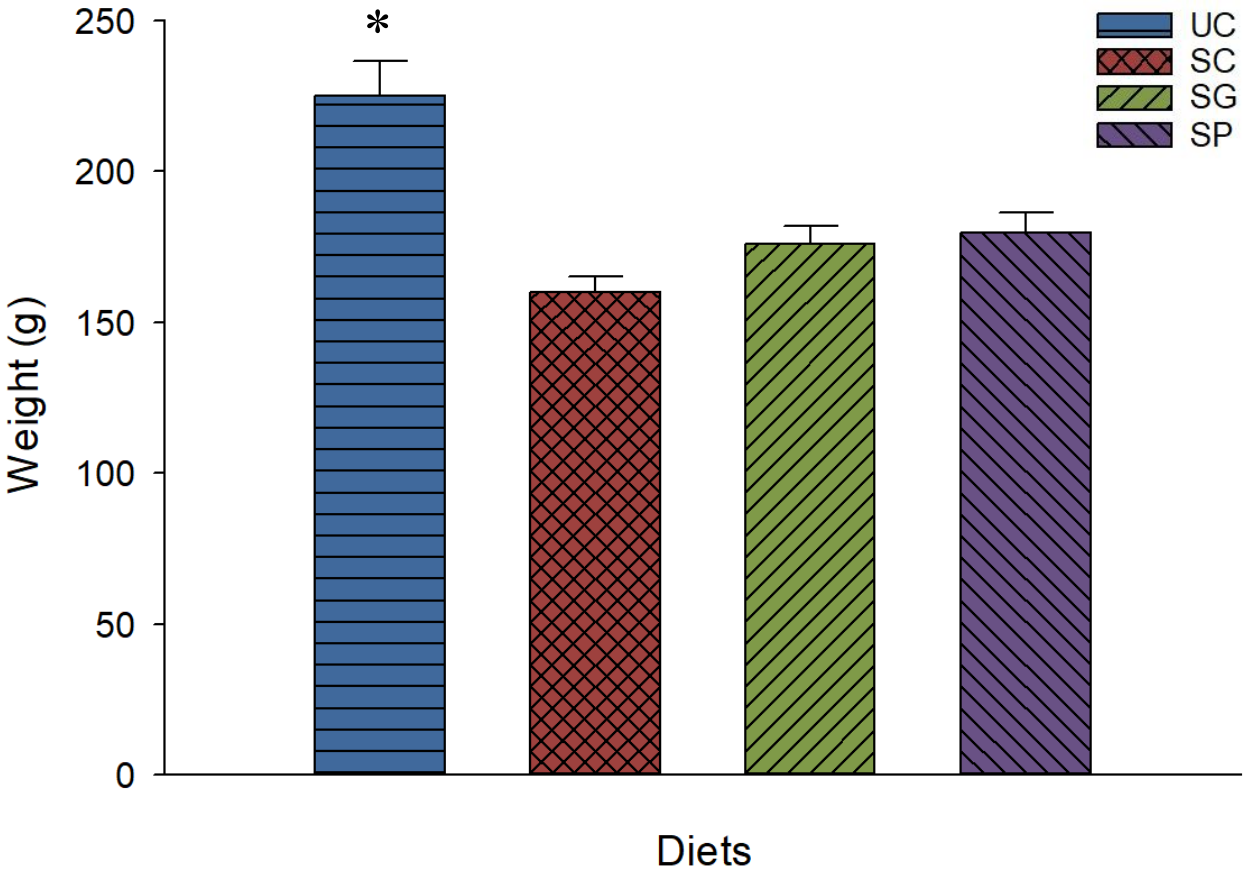

Figure 14: Effect of diets on terminal (day 49) weight. UC = unstressed fish fed commercial feed, SC = stressed fish fed commercial feed, SG = stressed fish fed genistein-supplemented feed, SP = stressed fish fed puerarin-supplemented feed. Bars represent mean + SEM; * = $p < .05$ compared to SC at day 49.

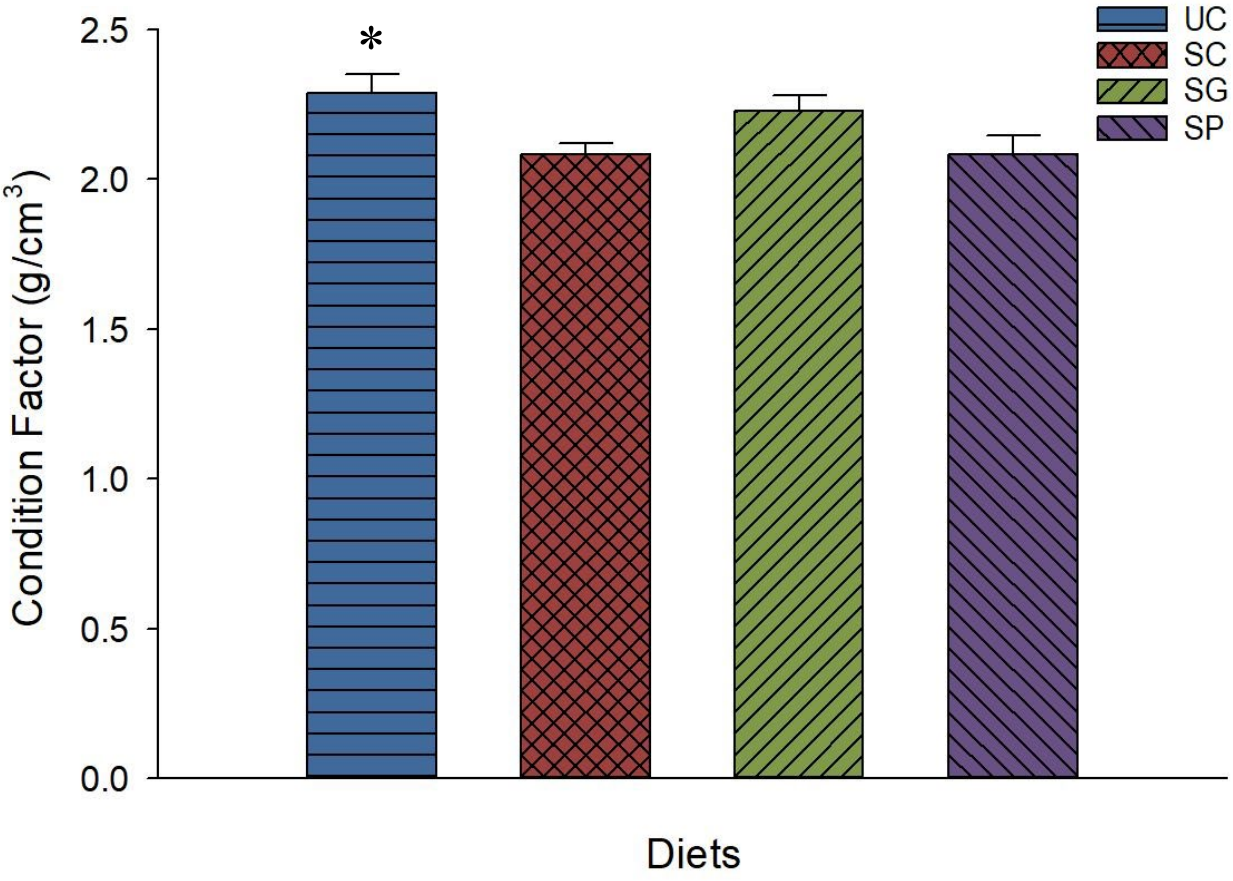

Figure 15: Effect of diets on terminal (day 49) condition factor. UC = unstressed fish fed commercial feed, SC = stressed fish fed commercial feed, SG = stressed fish fed genistein-supplemented feed, SP = stressed fish fed puerarin-supplemented feed. Bars represent mean + SEM; * = $p < .05$ compared to SC at day 49.

Table 5: Effects of diets on B_Δ, W_Δ, SGR, FI, FCR, and PER over 49 weeks. UC = unstressed fish fed commercial feed, SC = stressed fed commercial feed, SG = stressed fish fed genistein-supplemented feed, SP = stressed fish fed puerarin-supplemented feed. Values represent mean ± SEM; * = $p < .05$ compared to SC.

Growth Parameters	**UC**	**SC**	**SG**	**SP**
Biomass (g)				
B_i	566.3	566.3	566.3	566.3
B_f	1350.2	961.1	1077.1	1056.7
Daily B_Δ	16.00	8.06	10.42	10.01
Total B_Δ	783.9	394.8	510.8	490.4
Weight (g)				
W_i	94.4 ± 3.2	94.4 ± 3.2	94.4 ± 3.2	94.4 ± 3.2
W_f	225.0 ± 11.5*	160.2 ± 5.0	176.1 ± 5.8	179.5 ± 7.0
Daily W_Δ	2.67	1.34	1.74	1.67
Total W_Δ	130.7	65.8	85.1	81.7
SGR (g)	1.77	1.08	1.31	1.27
FI (g)				
Daily FI	17.3	15.3	15.4	15.2
Total FI	846.6	748.2	752.4	742.8
FCR	1.08	1.90	1.47	1.51
PER	1.85	1.06	1.36	1.32

Discussion

There is a lag in the production of corticosteroids, making cortisol an effective measurement of overall stress, even after the capturing and handling required to sample fish (Barton, 2002). While an overall decrease in cortisol may appear counterintuitive, the addition of cortisol to the diets (in the form of hydrocortisone) affects the negative feedback mechanism of stress (Barton, 2002; Barton et al., 1987). The HPI axis is controlled through negative feedback, and the repeated exposure of stress causes desensitization of the HPI axis, resulting in a decreased production of cortisol (Barton, 2002; Barton et al., 1987; Madaro et al., 2015). The mechanism of this negative feedback mechanism is twofold: 1) increased cortisol metabolism, leading to more rapid clearance of cortisol from the system (Redding et al., 1984), and 2) chronic stress leads to downregulation of tissue cortisol receptors (Pottinger, 1990, Barton et al., 1987). Specifically, the chronic administration of cortisol shuts down release of corticotrophic-releasing factor from the hypothalamus, which downregulates the production of adrenocorticotrophin from the anterior pituitary (Barton et al., 1987; Basu et al., 1965). The lack of adrenocorticotrophin stimulating interrenal chromaffin cells to produce cortisol leads to atrophy of the interrenal tissue (Barton et al., 1987; Basu et al., 1965). As shown in this study, hydrocortisone administration to Nile tilapia resulted in downregulation of serum cortisol concentration, demonstrating a lower production of endogenous cortisol production, as measured 24 after final administration of hydrocortisone. Stressed fish showed a significant decrease in endogenous cortisol production compared to unstressed fish. Even so, the addition of hydrocortisone into the feed still affects target tissues, producing negative consequences on growth and immunity (Barton et al., 1987). These findings show that the cortisol administered to the diets was indeed metabolized by the stressed fish, and was expected to cause negative impacts on physiology and growth. Although not statistically significant, the supplementation of pure isoflavone isolates in the feed showed overall higher cortisol concentrations, suggesting their use may alleviate the negative impacts of cortisol administration and produce a suppressed stress response.

Under stress, fish release blood glucose to help meet increased energy demands, resulting in an increased blood glucose concentration (Barton et al., 1987). This study did not demonstrate a significant increase in blood glucose concentration for stressed fish. Genistein supplementation showed significantly increased blood glucose concentration in the early stages of stress, although

this appeared to be eliminated or even reversed in the later stages of stress. One possible mechanism for the increase in blood glucose concentration is through the upregulation of *insig1* gene by genistein (Olsvik et al., 2017). This gene produces a membrane protein embedded in the endoplasmic reticulum membrane, and plays a role in many physiological processes, including glucose metabolism (GeneCards Database). Genistein was also shown to modulate the glycolysis pathway, decreasing the glucose-6-phosphate intermediate (Olsvik et al., 2017). Genistein supplementation also resulted in an increased HSI, although this was not significant. The liver is the main carbohydrate storage center in animals, with glucose being converted into glycogen and stored in the liver (Wendelaar Bonga, 1997). This increase in liver size associated with genistein may be due to decreased breakdown of glycogen into glucose. Genistein was shown to inhibit glycogenolysis, downregulating many of the pathway end products, such as maltose (Olsvik et al., 2017). Although genistein was shown to increase blood glucose concentrations, it is possible that molecules other than glycogen are being utilized in glucose production. The findings of this study suggest genistein supplementation may negatively impact glucose metabolism and homeostasis in Nile tilapia. Conversely, puerarin did not demonstrate any impacts on blood glucose or liver size.

Stress caused an overall increase in hematocrit, increasing the number of red blood cells circulating in the blood, although this was only statistically significant at day 21. The release of epinephrine and norepinephrine during stress results in the release of additional red blood cells and swelling of those red blood cells (Wendelaar Bonga, 1997). Genistein supplementation was able to prevent this release of red blood cells in the early stages of stress. Both genistein and puerarin consistently showed non-significantly lowered hematocrit values throughout the duration of the experiment, further suggesting these isoflavones may reduce the negative consequences of stress on circulating red blood cells.

Induction of stress in Nile tilapia produced changes in total plasma protein concentration. Stress caused an overall increase in plasma protein concentration, with a rise in protein concentration being continuous with the duration of stress. Increase in plasma protein concentration is thought to be an indicator of good health (Panigrahi et al., 2010), but an increase in plasma protein is also associated with stress (Ellis et al., 2012). This rise in plasma protein concentration is thought to

be related to immunity (Dawood et al., 2017; Gerwick et al., 2002). Injection of a variety of bacteria and viral factors caused on overall increase in plasma protein concentration (Gerwick et al., 2002). While an overall increase was shown, the protein profiles (as determined by protein molecular weight) showed upregulation of individual, un-identified proteins varied between different bacteria and different viral factors (Gerwick et al., 2002). During the early stages of stress, genistein had lower plasma protein concentration while puerarin showed increased plasma protein levels. As the duration of stress progresses, this trend changed, with genistein having higher plasma protein concentrations and puerarin having lower plasma protein concentration. A study that has looked into the effects of puerarin on plasma protein concentration found similar results, showing an overall decrease in plasma protein concentration (Hossain et al., 2013). Without proper controls in place, it is difficult to draw conclusions about whether an additional increase in plasma protein concentration above that caused by stress alone should be considered a positive or negative influence. While the increase in plasma protein concentration may indicate more robust innate immunity, it may also be due to an increase in stress. Generally speaking though, increased protein concentration would require additional energy expenditure to produce said proteins, suggesting further deviations from homeostasis.

Stress also showed a non-significant decrease in spleen size, an important organ that plays a role in adaptive immunity. Spleen size has shown to be predictive of disease resistance in fish, but the exact mechanism has not been determined (Hadidi et al., 2008). It is possible that increased spleen size allows for more efficient filtration of the blood or that spleen size is directly related to the composition of spleen cells (Hadidi et al., 2008). Overabundance or deficiencies in specific spleen cells may be responsible for the related spleen size (Hadidi et al., 2008). Stress is known to cause apoptosis of circulating lymphocytes and lymphocytes in the spleen (Saha et al., 2003). Fish lymphocytes also contain catecholamine receptors, leading to the hypothesis that lymphocytes can be directly innervated by the autonomic nervous systems (Nardocci et al., 2014), possibly mediated through the stress response. The addition of pure isoflavones resulted in an increase in spleen somatic index, but this was only significant for puerarin. Other research findings also suggest there is a positive effect of puerarin on spleen somatic index (Hossain et al., 2013), supporting the finding that puerarin significantly increases spleen size.

Negative consequences of stress on growth were apparent in this study. Stressed Nile tilapia showed overall lower growth rates, indicated by a lower specific growth rate and lower weight and biomass gains. Stress also resulted in leaner fish, as indicated by a significantly lower condition factor. Stress is thought to reduce growth in a number of different ways. First, stress leads to a decrease in feed intake (Wendelaar Bonga, 1997), which was simulated in this study. Stressed fish showed a lower daily and total feed intake compared to non-stressed fish. Neither genistein nor puerarin appeared to caused changes in overall feed intake. Second, stress causes negative consequences on the assimilation of food into body weight (Wendelaar Bonga, 1997). Again, stress showed much greater feed conversion ratio compared to non-stressed fish, meaning it required larger quantities of feed to produce increases in weight gain. While the isoflavones supplemented in the diets did not appear to influence feed intake, there is evidence to suggest that genistein and puerarin did influence the assimilation of food, requiring less food than the stressed fish that did not receive isoflavone supplementation. Stressed fish also had a lower protein efficiency ratio, indicating that stress resulted in larger quantities of dietary protein to produce growth. Again, genistein and puerarin supplemented to the diets appeared to alleviate the consequences of stress, as demonstrated by less protein being required in the diets. Lastly, stress increases overall metabolic rate above basal metabolism (Wendelaar Bonga, 1997). Metabolic rate can be examined in a number of different ways, but one prime example is the rate of respiration (Wendelaar Bonga, 1997). Research has shown that respiration rate is negatively associated with growth in fish, where an increase in respiration, and consequently an increase in metabolism, leads to an overall reduction of growth (Vaughan et al., 1982). Increases in respiration rates have been associated with increases in stress (Barton et al., 1987). Since the rate of metabolism was not directly examined in this study, no conclusions about metabolic rate can be drawn from this study.

Little to no information regarding the effects of puerarin on growth is available. Genistein has been well studied, but there are conflicting results regarding the effects of genistein on growth. Low doses of genistein have been shown to have no effect of growth on flounder, but genistein did show inhibitory effects on growth at greater concentrations (DiMaggio et al., 2016). Yellow perch have shown decreased growth at concentrations of genistein nearly four-fold greater than what was tested in this study (Ko et al., 1999). Other studies have found no effect of genistein on

growth in trout (Bennetau-Pelissero et al. 2001) or in bass (Pollack et al., 2003). One study found that the addition of genistein to the feed resulted in a decreased feed intake in trout, but it also produced a greater protein utilization (Torno et al., 2018). This partially supports the findings of this research. While genistein supplementation did not decrease feed intake, it did result in greater protein efficiency, possibly due to protein utilization as described by Torno et al. (2018).

Conclusion

The effects of stress on fish causes many deleterious effects. The isoflavones genistein and puerarin appear to alleviate some of these negative consequences, such as the effects of stress on red blood cell release and plasma protein concentration. These negative consequences of stress are due to the release of catecholamine and corticosteroids, such as cortisol. The use of cortisol added to the feed allowed for downregulation of endogenous cortisol production while still producing the negative consequences of stress. One possible negative consequence of genistein supplementation is an imbalance in glucose homeostasis, which may be explained due to the effects of genistein on the glycolysis and glycogenolysis pathway. Increases in spleen size shows that puerarin and possibly genistein promote the adaptive immune system. Genistein and puerarin also produced differing effects on plasma protein concentrations, although further investigation is needed to determine the relationships between stress, isoflavones, and this innate immune response. These isoflavones also showed overall better growth rates in Nile tilapia, as seen with a greater specific growth rate, weight gain, feed conversion, and protein efficiency, all while requiring the same amount of feed. While further research is needed to better understand the effects of isoflavone on stress, these findings suggest that isoflavones may be effective modulators of the stress response.

COMPARISON OF CRUDE ISOFLAVONE EXTRACTS ON STRESS

Introduction

Aquaculture, a rapidly growing source of high quality protein (Troell et al., 2014), has the potential to feed millions of people and help reduce world hunger. Unfortunately, this growth is coming at the expense of the fish being reared. Crowded conditions and other stressors in this artificial environment results in the release of catecholamines and corticosteroids, which inhibit growth and immunity (Barton, 2002). This results in huge economic losses as fish succumb to disease and death (Liu et al., 2017). To counteract these side effects, antibiotics and other chemicals are used prevalently and with little discrimination (Hvistendahl, 2012, Watts et al., 2017), promoting antibiotic resistance and passing antibiotic residues along to consumers (Caruso, 2016). Nutraceuticals may help turn this tide by taking a more proactive response to stress. Isoflavones are of particular interest. They are naturally abundant in some widely consumed foods (Ganai & Farooqi, 2015) and have many different health benefits associated with them. Although structurally similar, isoflavones do not share all of the same health benefits (Ruiz-Larrea et al., 1997; Yanagihara, 1993), and different isoflavones may have more potent health effects compared to others.

In nature though, these isoflavones are not produced in isolation. Legumes produce many different isoflavones, with variations in the relative abundance of these isoflavones between plants. Burdette and Marcus (2013) found isoflavones composition varies greatly between different legume dietary supplements. Soybean (*Glycine max*) flour and soybean protein isolate showed genistein in the greatest concentration, closely followed by daidzein. Commercially available soybean products do not appear to follow this same trend in isoflavone composition. Daidzein is the isoflavone found in greatest abundance in commercial soybean (*Glycone max*), followed by genistin and glycitin (Chen and Wei, 2008). Converting soybean into other soy products appears to produce changes in the relative abundance of these isoflavones. In soymilk and tofu, genistin was the most abundant isoflavone, followed by daidzein then glycitin (Chen and Wei, 2008). Further complicating this issue, isoflavone composition can vary greatly within the same plant. The soybean germ was shown to have the greatest concentration of isoflavone,

with daidzin and glycitin being produced in the greatest abundance (Yue et al., 2010). Although at lower concentration, the soybean coat contained the same relative abundance of isoflavones (Yu et al., 2010). Daidzin was the most abundant isoflavone in the soybean cotyledon, but instead of glycitin, genistein was the second most abundant product. Isoflavone concentration also varied greatly within and between clover species (Butkute et al., 2014). Red clover (*Trifolium pretense*) flower and red clover extract contained mostly formononetin and biochanin A (Burdette and Marcus, 2013). Formononetin was generally the most abundant isoflavone across species and tissues, although there was great variation in the second most abundant isoflavone (Butkute et al., 2014). Puerarin and daidzein were the most abundant isoflavone in kudzu (*Pueraria lobate*) rhizome and extract products (Burdette and Marcus, 2013). Other studies have supported this finding (Reppert et al., 2008), suggesting there may be little variations in isoflavone composition in kudzu. Lastly, dietary supplements are not always sold on a plant-by-plant basis either, but may combine a variety of isoflavone-producing plants. As expected, there are large variations in isoflavone composition across different dietary supplements containing mixtures of these legumes (Delmonte et al., 2006). These findings clearly demonstrate that relative isoflavone abundance can vary greatly between different isoflavone-containing plants and their products, both between species and even within species.

While many studies have tried to tease apart the individual effects of isoflavones (Ruiz-Larrea et al., 1997; Yanagilhara, 1993; Rasbach & Schnellmann, 2008), the holistic consumption of isoflavones from legumes also needs to be considered. That is, there may be combined effects of isoflavones when used in conjunction with one another that is different than any one individual effect. The nature of those combined effects is not well understood, although a few recent studies have worked to better understand these effects. Huang et al. (2016) found that soybean extract containing a crude isoflavone mixture produced a decrease in macrophage nitrate production, a precursor to respiratory burst. While the individual effects of genistein and daidzein were able to stimulate a similar reduction in nitrate production, those individual effects were significantly different than those produced from the crude isoflavone extract. Wu et al. (2019) took a different approach by mixing individual isoflavones together to create isoflavone concentrations identical to those in crude isoflavone extracts from soybean. They found that crude soybean isoflavone extracts produced increases in growth and changes in oocyte histology in fish, but the individual

isoflavone mixture did not produce the same results, even though these isoflavones were standardized to the crude isoflavone extract. While more research is needed in this area, this suggests that crude isoflavone extracts may produce substantially different results than their individual components.

The objective of this experiment was to determine the effects of three crude isoflavone extracts (kudzu, red clover, and soybean) on stress in Nile tilapia. It was hypothesized that kudzu, red clover, and soybean isoflavone extracts will reduce stress, resulting in increased growth and improved immunity (Table 6).

Method

Fish Acquisition and Maintenance

Fingerling Nile tilapia (*Oreochromis niloticus*) (n=120) obtained from Troyer Aqua Farm (Indiana, USA) were reared in a recirculating system in the Life Science Resource Center on the Purdue University Fort Wayne campus. One hundred twenty fish were randomly distributed into 15 20-gallon tanks (n=8), each fed their own unique diet (Fig. 16). Twenty percent water changes were performed on a weekly basis for the duration of the experiment, with water chemistry being measured on a weekly basis. Water chemistry was maintained at a healthy level (ammonia < 0.25 ppm, nitrite < 0.5 ppm, nitrate < 40 ppm, dissolved oxygen > 7.0 mg/L). The pH and temperature ranged from 7.2-7.6 and 25-28°C, respectively. A photoperiod of 14-hour daylight, 10-hour night was maintained throughout the duration of the experiment.

Table 6: Hypothesized results of stress on various physiological and growth parameters. Supplementation of isoflavones for stressed fish is expected to improve these parameters.

Parameter	Stress	Isoflavone Supplementation
Serum Cortisol	↓	↑
Blood Glucose	↑	↓
Hematocrit	↑	↓
Plasma Protein	↑	↓
Hepatosomatic Index	↓	↑
Spleen-Somatic Index	↓	↑
Length	-	-
Weight	↓	↑
Biomass	↓	↑
Specific Growth Rate	↓	↑
Feed Intake	↓	↑
Feed Conversion Ratio	↑	↓
Protein Efficiency Ratio	↓	↑
Condition Factor	↓	↑

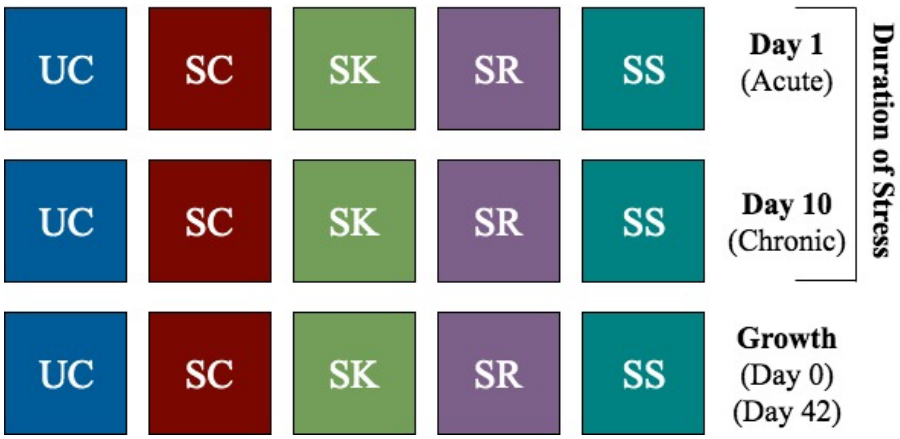

Figure 16: Study design. Fish from each of the five diets were sampled four times. Acute and chronic stress was measured at days 1 and 10, respectively. Growth was measured at day 0 and 42. UC = unstressed fish fed commercial feed, SC = stressed fish fed commercial feed, SK = stressed fish fed kudzu-supplemented feed, SR = stressed fish fed red clover-supplemented feed, SS = stressed fish fed soybean-supplemented feed.

Diets

AquaMax® Fingerling Starter 300 commercial feed was obtained from Purina® (Missouri, USA), with nutritional information provided in Table 7. Fish were fed twice daily at 1.5% of mean body weight, with fish being weighed on a weekly basis to determine mean body weight. Hydrocortisone was used to induce stress in fish, which has been reported previously (Barton, et al, 1987). To stress fish, 100 mg of hydrocortisone, 98% (Acros Organics, New Jersey, USA), dissolved in pure ethanol was mixed with one kg of commercial feed in an enclosed container. Feed pellets were spread out on a tray and allowed to air dry overnight before use. Crude isoflavone extract was added to the feed in a similar fashion as hydrocortisone, with the exception that 5 g of 40% crude isoflavone extract was added per kg of commercial feed, to create a concentration of 2 g of isoflavone per kg of feed. Feed was placed in a sealed container and stored at 4ºC. 40% crude isoflavone extracts of kudzu (*Pueraria lobata*), red clover (*Trifolium pratense*), and soybean (*Glycine max*) were obtained from Nutra Green Biotechnology Co. (California, USA). Individual isoflavone composition of the 40% crude extracts was provided upon purchase (Table 8).

Experimental Design

Fish were allowed to acclimate for two weeks prior to starting the experiment, during which time they were fed commercial feed until satiation. Fish were fed one of the five experimental diets: 1) unstressed fish fed commercial feed (UC), which served as a negative control for stress, 2) stressed fish fed commercial feed (SC), which served as a positive control for stress, 3) stressed fish fed kudzu extract-supplemented feed (SK), 4) stressed fish fed red clover extract-supplemented feed (SP), and 5) stressed fish fed soybean extract-supplemented feed (Fig. 16). There were three tanks for each of the five diets. One set of tanks with eight fish from each of the five diets was sampled at day 1, which constituted the acute stress portion of the physiology study, and at day 10. The second set of tanks with eight fish from each of the five diets was sampled at day 10, which constituted the chronic stress portion of the study. The third set of tanks containing eight fish was utilized for the growth study. Fish from each of the five diets were collected at day 0 (baseline), before the diets were administered. Length and weight were recorded for each fish. The same fish from the five tanks were again measured 42 days after

feeding with the experimental diets. The growth portion of this study represented a repeat-measure design, but the physiology portion did not.

Fish were euthanized by adding 200 mg of Tricaine-S (MS-222) (Western Chemical, Washington, USA) to one liter of water, waiting until operculum movement and response to stimuli ceased. Weight and length were collected at every sampling period. At day 1 and day 10, blood was collected from the caudal vein of each fish using a heparinized (Sequester-Sol, Florida, USA) 25-gauge needle (BD Syringe, New Jersey, USA). To obtain plasma, blood was immediately centrifuged at 10,000 rpm for 5 minutes before clotting occurred. To obtain serum, blood was allowed to sit on ice for one hour so clotting could occur. After clotting, the blood was spun at 5000 rpm for 10 minutes using a centrifuge. Excess serum was stored at -80ºC until ready for use. Fish were then dissected to collect the liver and spleen.

Physiological Parameters

Serum cortisol concentration was analyzed using a commercial cortisol enzyme-linked immunosorbent assay (ELISA) kit purchased from Cayman Chemical (Michigan, USA). Procedure was followed according to manufacturer's guidelines (Cayman, 2017). Briefly, serum was removed from -80ºC and allowed to thaw before use. First, ELISA buffer was added to a 96-well plate, followed by the standard cortisol concentrations or serum sample. Next, cortisol acetylcholinesterase (AChE) inhibitor was added before finally adding cortisol ELISA monoclonal antibodies. Plates were covered in plastic film and allowed to incubate overnight at 4ºC. After incubation, plates were emptied and rinsed five times using wash buffer. Ellmen's reagent was added to each well, plates were covered with plastic film, and incubated at room temperature for 90 minutes using an orbital shaker. Plates were read at 420nm using a Multiskan GO (Thermo Scientific, Massachussets, USA) plate reader. Data was analyzed using a four-parameter logistic fit with log concentration of cortisol plotted on the x-axis and $\%B/B_0$ plotted on the y-axis.

Blood glucose concentration was determined using heparinized blood. One drop of blood from each sample was added to a FreeStyle Lite (Abbott, California, USA) blood glucose test strip and read using a FreeStyle Lite (Abbott, California, USA) glucometer. The FreeStyle Lite

glucometer has shown to be one of the most accurate glucometers on the market, with a 96% compliance rate (Klonoff et al., 2018).

To determine hematocrit (packed cell volume), heparinized blood was added to a capillary tube, sealed at one end using a critocap, and spun at 10,000 rpm for five minutes using a microcentrifuge. The capillary tube with separated blood was placed on a microhematocrit capillary tube reader and the percentage of red blood cells was read.

Plasma protein concentration was determined using a VEE GEE CLX 1 (Washington, USA) refractometer. 2 drops of plasma were added to the surface of the prism. The plate lid was closed and placed under a bright light to view the blue-white boundary, indicative of the plasma protein concentration. The refractometer was properly cleaned between samples.

Lysozyme activity was measured according to Ellis (1990). Briefly, the lyophilized gram-negative bacterium *Micrococcus lysodeikticus* (Sigma, Missouri, USA) was weighed and added to sodium phosphate buffer (0.05M, pH 6.2) to create a suspension of 0.2 mg of bacteria per mL. Serum (25 µL) was added to 1.0 mL of *M. lysodeikticus* suspension and briefly vortexed. Absorbance (530 nm) was recorded at 30 seconds (Abs_I) and 4 minutes 30 seconds (Abs_F) using a Milton Roy Spectronic 601(Pennsylvania, USA) spectrophotometer. Lysozyme activity was calculated using the equation below. A unit of lysozyme activity is described as causing a decrease in absorbance of 0.001 per minute.

$$\frac{\mathrm{Abs_I} - \mathrm{Abs_F}}{\mathrm{min}}$$

To determine the organ-somatic indices, both the spleen and liver were weighed after dissection. Hepatosomatic index (I) and spleen-somatic index (SSI) were calculated by the following equation:

$$\text{HSI: } \frac{\text{Liver weight (g)}}{\text{Total body weight (g)}} * 100 \qquad \text{SSI: } \frac{\text{Spleen weight (g)}}{\text{Total body weight (g)}} * 100$$

Growth Parameters

Initial length (L_I) in cm and initial weight (W_I) in g were collected at day 0. Initial biomass (B_I) in g was calculated by the addition W_I from the eight-fish sample. Using the same eight fish from initial measurements, final length (L_F) in cm and final weight (W_F) in g were collected at day 42. Final biomass (B_F) in g was calculated by the addition W_F from the same six-fish. Condition factor was calculated as followed: [weight/length^3]. Total feed intake (FI) in g was calculated by the addition of feed added to the tank from each of the six weeks of sampling. Protein intake (PI) in g was calculated by multiplying FI by the proportion of protein in the feed. All growth parameters were calculated according to Table 9. Fish were assumed to homogenous growth, where the largest fish in the initial sample was compared with the largest fish in the final sample, and so on for the remainder of the fish.

Statistics

All physiological parameters and the growth parameters length, weight, and FCR were analyzed using a two-way (5x2) multivariate analysis of variance (MANOVA) using diets and stress response as the independent variables ($\alpha = .05$). The growth parameters TWG and SGR were analyzed by a one-way MANOVA using diets as the independent variable. The two-way MANOVA was followed-up using a series of univariate two-way ANOVAs for dependent variables that had a statistically significant interaction, examining diets at every level of stress response. Dunnett's test (many-to-one comparison) was used for all post-hoc analysis, with SC serving as the diet with which the other diets were compared. Comparing all other diets to only SC was utilized for two reasons: 1) SC is the only diet that has one degree of commonality with all other diets regarding stress and isoflavone supplementation, and 2) to minimize the type 1 (family-wise) error rate due to fewer overall comparisons than testing all possible differences between diets. If there was not a significant interaction for the dependent variable, only the statistically significant main effect of diets was examined using a univariate one-way ANOVA followed by Dunnett's test. Figure 17 provides a graphical summary of the statistical analyses used.

Table 7: Nutritional composition for experimental diets (expressed as % of total feed). UC = unstressed fish fed commercial feed, SC = stressed fish fed commercial feed, SK = stressed fish fed kudzu-supplemented feed, SR = stressed fish fed red clover-supplemented feed, SS = stressed fish fed soybean-supplemented feed.

Diets	UC	SC	SK	SR	SS
Crude Protein (%)	50.00	50.00	50.00	50.00	50.00
Crude Fat (%)	16.00	16.00	16.00	16.00	16.00
Crude Fiber (%)	3.00	3.00	3.00	3.00	3.00
Ash (%)	12.00	12.00	12.00	12.00	12.00
Calcium (%)	2.55	2.55	2.55	2.55	2.55
Phosphorus (%)	1.30	1.30	1.30	1.30	1.30
Sodium (%)	0.60	0.60	0.60	0.60	0.60
Hydrocortisone (%)	-	0.01	0.01	0.01	0.01
Kudzu Extract (%)	-	-	0.50	-	-
Red Clover Extract (%)	-	-	-	0.50	-
Soybean Extract (%)	-	-	-	-	0.50

Table 8: Ratio of isoflavones in crude extracts of kudzu (*Pueraria lobata*), red clover *(Trifolium pratense*), and soybean (*Glycine max*)

Isoflavone	Kudzu	Red Clover	Soybean
Biochanin A	-	8.5%	-
Daidzein	3.3%	0.4%	1.4%
Daidzin	6.2%	-	21.8%
Formononetin	-	30.7%	-
Genistein	-	0.3%	0.3%
Genistin	-	-	4.5%
Glycitein	-	-	0.5%
Glycitin	-	-	11.4%
Puerarin	30.4%	-	-
Total	**40.0%**	**40.0%**	**40.0%**

Table 9: Equations used to determine growth parameters

Growth Parameter	Equation
Total biomass change (B_Δ)	$B_F - B_I$
Daily B_Δ	$\frac{B\Delta}{Days}$
Total weight gain (W_Δ)	$W_F - W_I$
Daily W_Δ	$\frac{W\Delta}{Days}$
Specific Growth Rate (SGR)	$\frac{\ln(W_F) - \ln(W_I)}{Days} * 100$
Daily FI	$\frac{Total\ FI}{Days}$
Feed Conversion Ratio (FCR)	$\frac{(FI/Fish\ in\ Sample)}{Total\ W\Delta}$
Protein Efficiency Ratio (PER)	$\frac{(PI/Fish\ in\ Sample)}{Total\ W\Delta}$
Condition Factor	$\frac{Weight}{Length^3}$

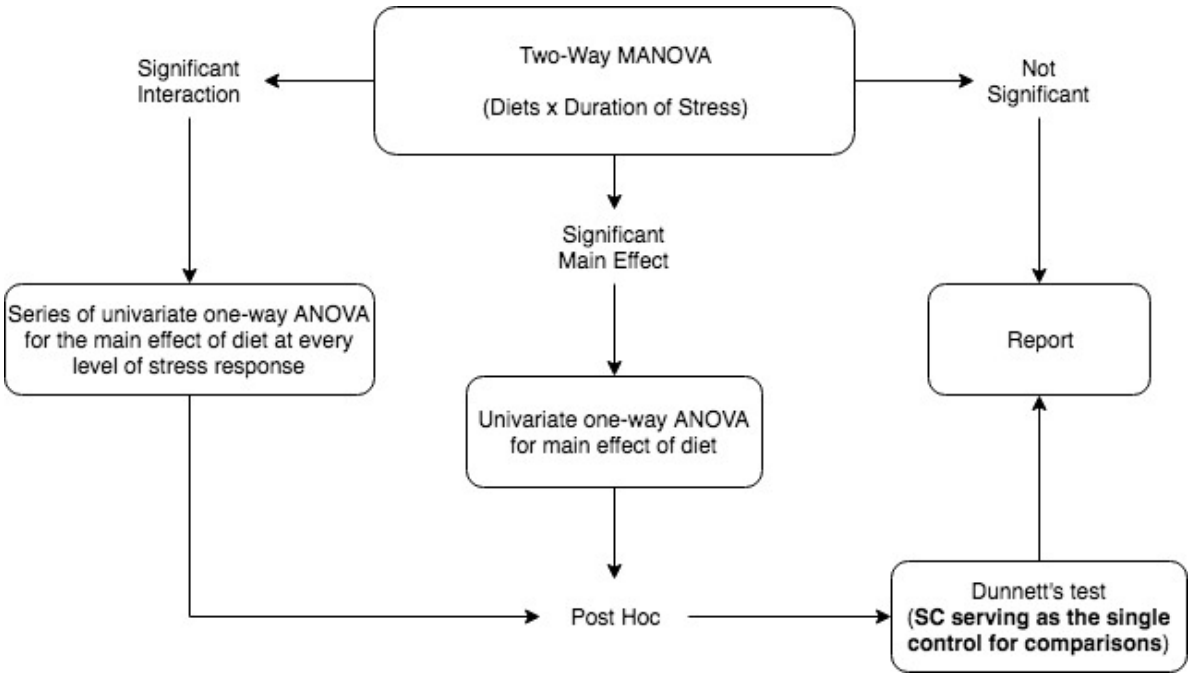

Figure 17: Flow chart for statistical analysis of diets and stress response on physiological and growth parameters

Results

The results of the MANOVA analyses are presented in Table 10. There was not a significant main effect of diets on serum cortisol concentration ($p = .134$) (Fig. 18), blood glucose concentration ($p = .865$) (Fig. 19), or hematocrit ($p = .151$) (Fig. 20), although it does appear that stress caused an overall decrease in serum cortisol concentration and hematocrit. There was a significant interaction between diets and stress response on plasma protein concentration ($p = .001$) (Fig. 21). When examining the effect of diets on stress, there was a significant main effect for diets during chronic stress ($F(4,35) = 8.232$, $p > .001$) but not acute stress ($F(4,33) = .776$, $p = .549$). During chronic stress, the addition of hydrocortisone to the feed appeared to elevate plasma protein levels, although there were no significant differences between UC and SC, which had a plasma protein concentration of 5.45 ± 0.29 ($p = .187$). SK and SR did not significantly differ from SC either ($p = .917$ and $p = .856$, respectively). SS, which had a plasma protein concentration of 6.90 ± 0.23, did show a significantly higher plasma protein concentration though when compared to SC ($p = .003$).

While the main effect of diets did significantly impact LA ($p = .004$), none of the diets were shown to be significantly different from SC, the diet which all other were compared with ($p = .905$ for UC, $p = .162$ for SK, $p = .438$ for SR, and $p = .447$ for SS) (Fig. 22). Stress appeared to cause an overall increase in lysozyme activity, with the crude isoflavone extract supplemented diets showing an additional increase in lysozyme activity. A similar trend was shown for hepatosomatic index, where there was a significant main effect of diets on hepatosomatic index ($p = .046$), but there was no significant difference detected between SC and UC ($p = .905$), SK ($p = .162$), SR ($p = .438$), or SS ($p = .447$) (Fig. 23). Like lysozyme activity though, hepatosomatic index appeared to increase overall for the four stress diets, with the three crude isoflavone extract supplemented diets showing the greatest HSI.

Lastly, diets did show a significant effect on spleen-somatic index ($p = .003$) (Fig. 24). SC had an overall decrease in spleen-somatic index, with a measurement of 0.278 ± 0.033 %, compared to UC, which had an overall spleen-somatic index of 0.435 ± 0.033 % ($p = .013$). All diets that were supplemented with isoflavone showed a significantly greater spleen-somatic index, which

appeared to be comparable to the UC. Overall spleen-somatic index was 0.463 ± 0.043 % for SK (p = .002), 0.439 ± 0.054 % for SR (p = .013), and 0.418 ± 0.036 % for SS (p = .021).

There was not a significant effect of diets on length (p = .099) (Fig. 25), but there was a significant effect interaction between diets and time on weight (p = .025) and condition factor (p = .047). Weight and condition factor did not significantly differ at baseline (F(4,35) = .055, p = .994, and F(4,35) = 2.044, p = .110, respectively). At day 42 though, diets significantly impacted both weight (F(4,35) = 2.684, p = .047) and condition factor (F(4,35) = 7.340, p < .001). UC had a greater terminal weight (139.5 ± 11.7 g) than SC (102.0 ± 11.5 g) (p = .047), but there were no significant differences between SC and SK (p = .992), SR (p = .963), or SS (p = .999) (Fig. 26). UC also showed a more favorable terminal condition factor (2.03 ± 0.07) than SC (1.80 ± 0.04) (p = .002), but again, there were no statistical differences between SC and SK (p = .979), SR (p = .773), or SS (p = .572) (Fig. 27).

Due to the repeated measures of growth, it is possible to statistically analyze the effects of diets on W_{Δ} and SGR. Both were shown to be significantly affected by diets (p =.001 and p < .001, respectively). In both cases, UC had a greater W_{Δ} (83.2 ± 9.2 g) (Fig. 28) and SGR (2.12 ± 0.12 g) (Fig. 29) than SC, which had a W_{Δ} of 47.8 ± 6.1 g and a SGR of 1.53 ± 0.11 (p = .002 and p < .001, respectively). Of all the crude isoflavone extract-supplemented diets, SR showed the greatest W_{Δ} and SGR compared to SC, but these were not statistically significant (p = .818 and p = .594, respectively). For W_{Δ}, compared to SC, neither SK (p = .964) or SS (p = .993) were shown to be significantly different. The same is true for SGR (p = .519 and p = .994, respectively).

Focusing on the qualitative growth data presented in Table 11, UC showed a higher daily and total gain in both B_{Δ} and W_{Δ}, as well as a higher SGR, compared to all stress groups. For those stress groups that received crude isoflavone extract-supplementation in their diets, there were mixed results on growth. SR showed a greater SGR, and daily and total B_{Δ} and W_{Δ} when compared to SC although this was not statistically significant. SS showed similar growth results across most growth parameters, but did show possible improvements in FCR and PER. SK showed the lowest growth, as suggested by a lower daily and total B_{Δ} and W_{Δ}, as well as SGR.

For FI, UC required more food to achieve such growth compared to the stress groups. SC required the second most feed, and all crude isoflavone extract-supplemented feeds required lower quantities of feed than SC. This resulted in SR and SS having a lower FCR and a higher PER than SC. Even though SK had a lower growth, it also had a lower FI, resulting in a comparable FCR and PER as SC. Collectively, UC showed more favorable values across all growth parameters (tank biomass, weight, SGR, FCR, and PER) except FI, compared to all of the stress diets. With the addition of crude isoflavone extract-supplementation in the feed, SR and SS showed more favorable values across all growth parameters compared to SC, while SK showed similar values to SC.

Table 10: MANOVA results for the interactive (Int.) effects of time and diet or the main effect (ME) of diet on the combined physiological and growth parameters.

Statistical Test	Wilks Λ	*F*	*p*
Two-Way MANOVA	.290	(40, 199) = 1.920	.002
Serum Cortisol (Int.)	-	(4,61) = 1.573	.193
Serum Cortisol (ME)	-	(4,61) = 1.831	.134
Blood Glucose (Int.)	-	(4,61) = 1.535	.203
Blood Glucose (ME)	-	(4,61) = 1.573	.865
Hematocrit (Int.)	-	(4,61) = 1.505	.212
Hematocrit (ME)	-	(4,61) = 1.750	.151
Plasma Protein (Int.)	-	(4,61) = 5.106	.001
Lysozyme Activity (Int.)	-	(4,61) = 0.454	.769
Lysozyme Activity (ME)	-	(4,61) = 4.331	.004
Hepatosomatic Index (Int.)	-	(4,61) = 1.169	.628
Hepatosomatic Index (ME)	-	(4,61) = 2.588	.046
Spleen-Somatic Index (Int.)	-	(4,61) = 0.825	.259
Spleen-Somatic Index (ME)	-	(4,61) = 4.542	.003
Length (Int.)	-	(4,61) = 0.825	.514
Length (ME)	-	(4,61) = 1.877	.126
Weight (Int.)	-	(4,61) = 2.949	.027
Condition Factor (Int.)	-	(4,61) = 2.529	.050
One-Way MANOVA	.469	(8,68) = 3.914	.001
Specific Growth Rate (ME)	-	(4,35) = 9.116	<.001
Weight Gain (ME)	-	(4,35) = 6.064	.001

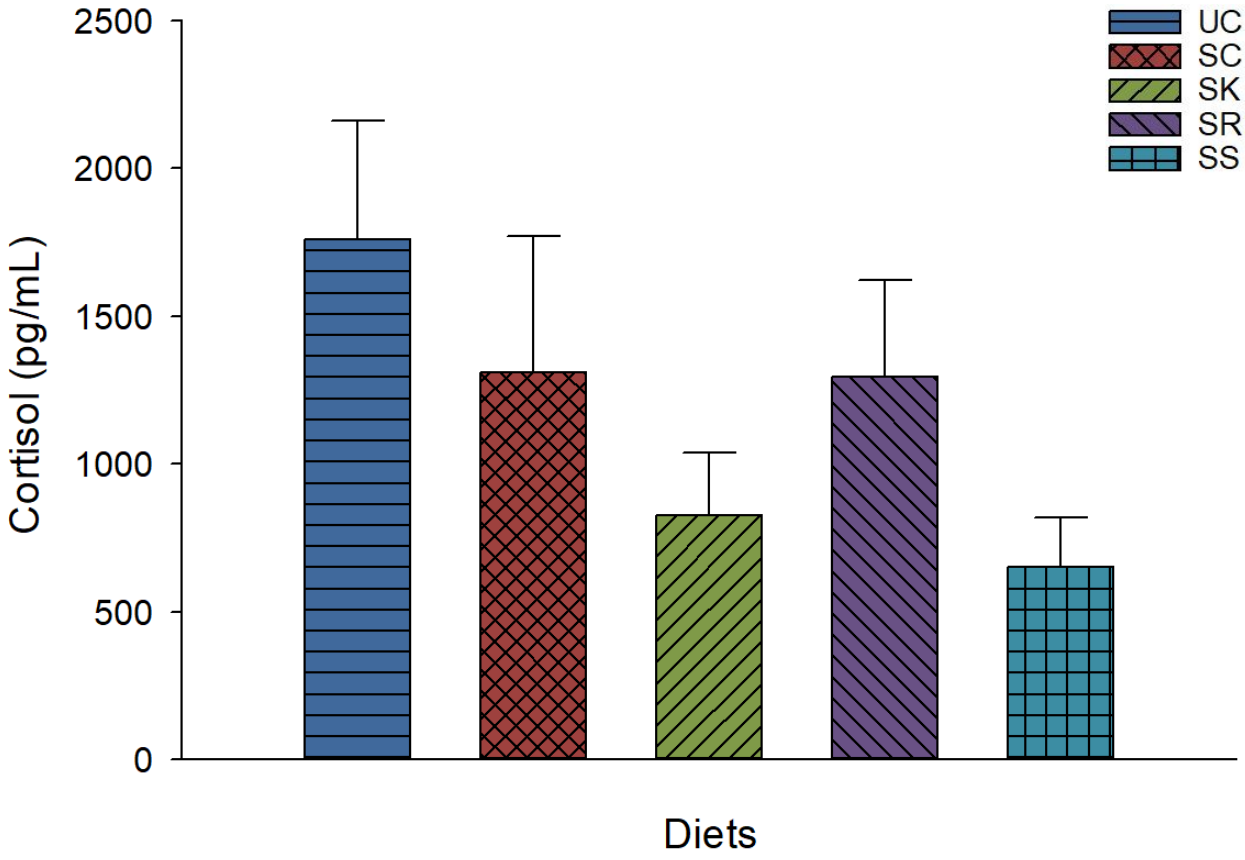

Figure 18: Overall main effect of diets on serum cortisol concentration. UC = unstressed fish fed commercial feed, SC = stressed fish fed commercial feed, SK = stressed fish fed kudzu (*Pueraria lobate*)-supplemented feed, SR = stressed fish fed red clover (*Trifolium pretense*)-supplemented feed, SS = stressed fish fed soybean (*Glycine max*)-supplemented feed. Bars represent mean + SEM.

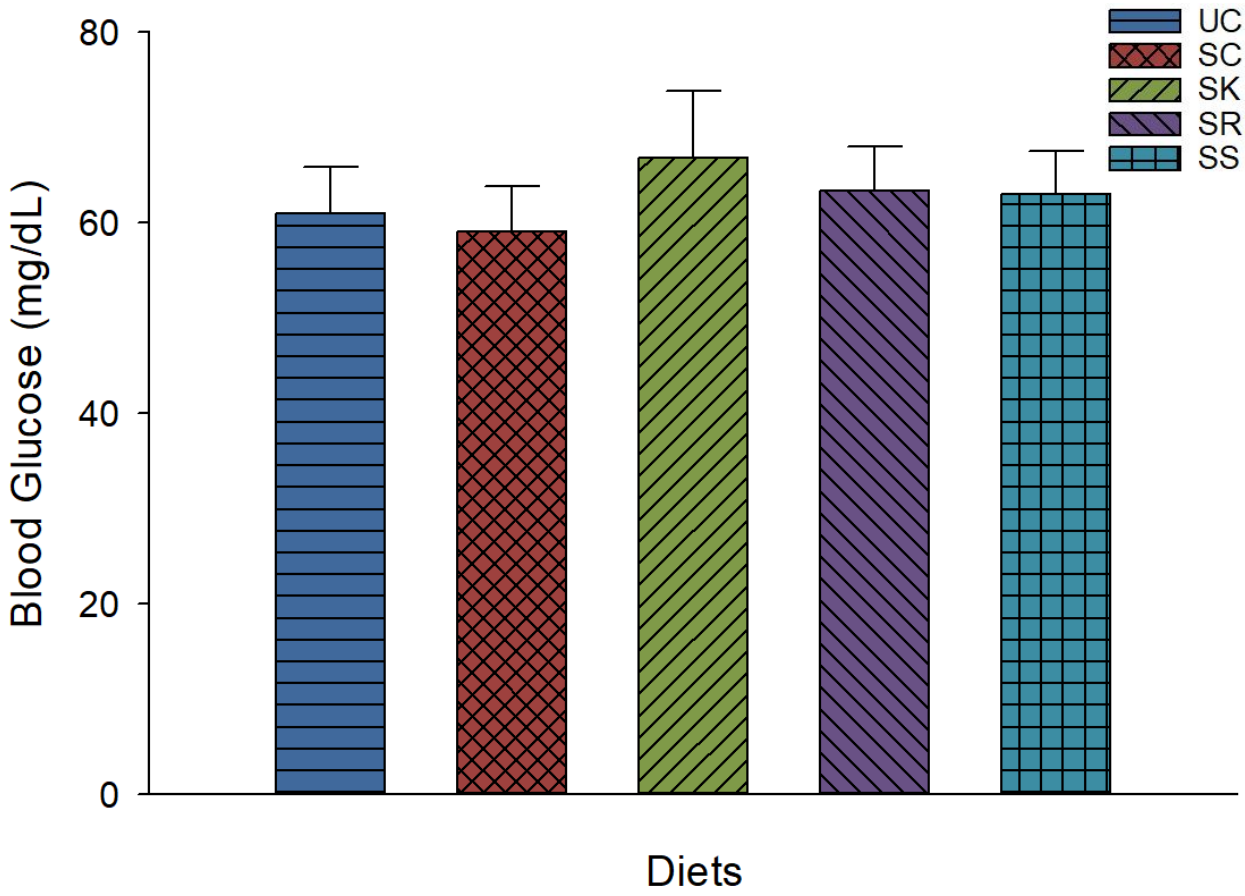

Figure 19: Overall main effect of diets on blood glucose concentration. UC = unstressed fish fed commercial feed, SC = stressed fish fed commercial feed, SK = stressed fish fed kudzu (*Pueraria lobate*)-supplemented feed, SR = stressed fish fed red clover (*Trifolium pretense*)-supplemented feed, SS = stressed fish fed soybean (*Glycine max*)-supplemented feed. Bars represent mean + SEM.

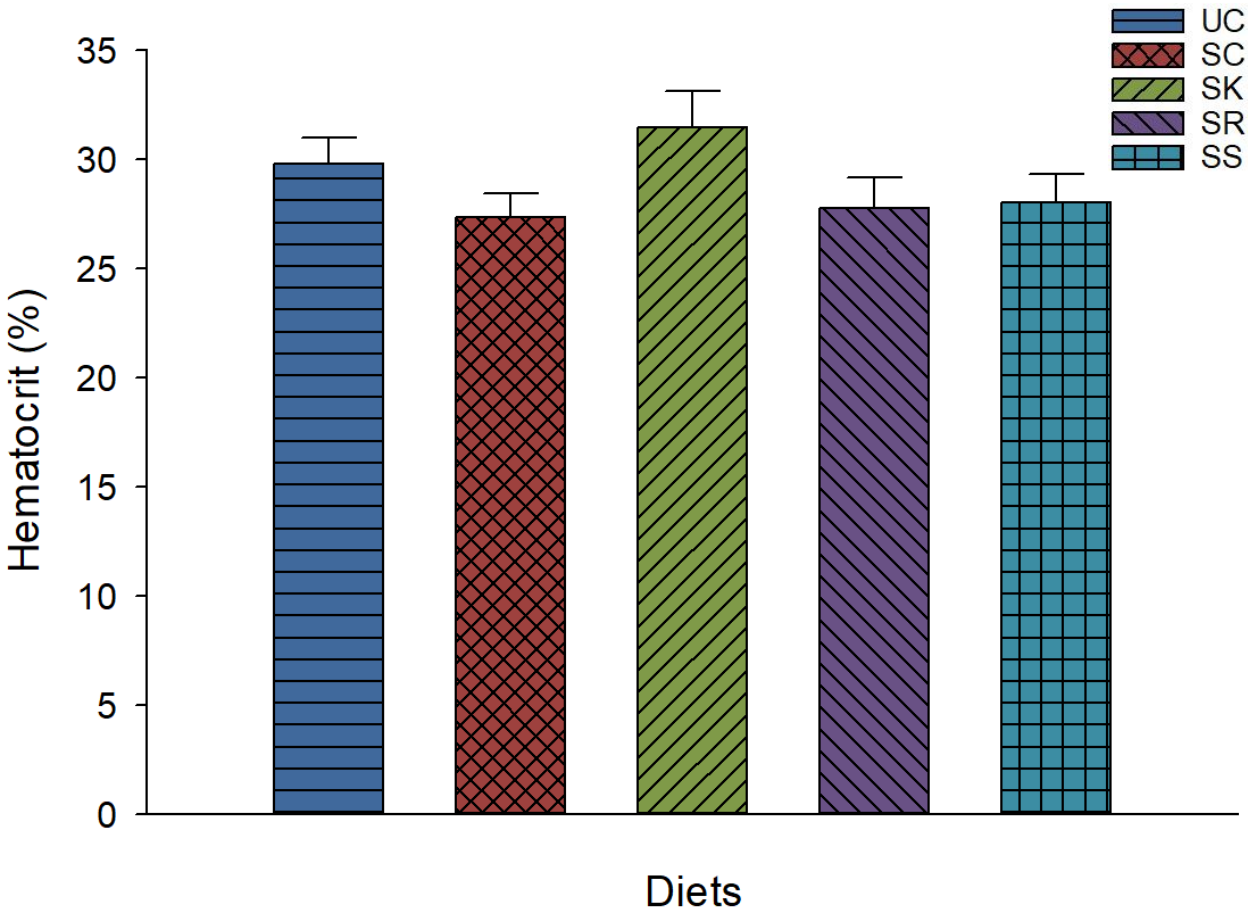

Figure 20: Overall main effect of diets on hematocrit. UC = unstressed fish fed commercial feed, SC = stressed fish fed commercial feed, SK = stressed fish fed kudzu (*Pueraria lobate*)-supplemented feed, SR = stressed fish fed red clover (*Trifolium pretense*)-supplemented feed, SS = stressed fish fed soybean (*Glycine max*)-supplemented feed. Bars represent mean + SEM.

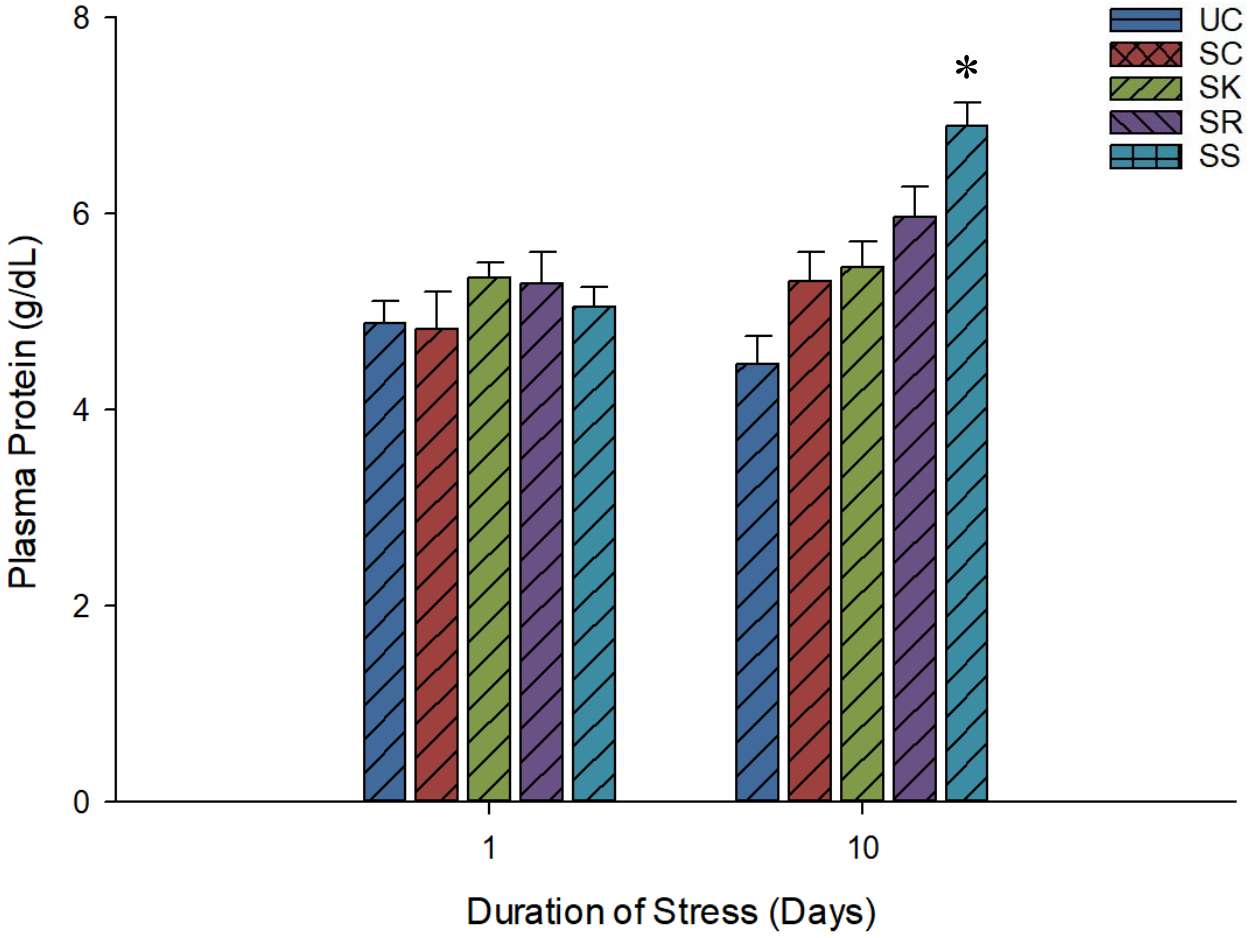

Figure 21: Effect of diets on plasma protein concentrations after day 1 and day 10 post-induction of stress. UC = unstressed fish fed commercial feed, SC = stressed fish fed commercial feed, SK = stressed fish fed kudzu (*Pueraria lobate*)-supplemented feed, SR = stressed fish fed red clover (*Trifolium pretense*)-supplemented feed, SS = stressed fish fed soybean (*Glycine max*)-supplemented feed. Bars represent mean + SEM; * = $p < .05$ compared to SC.

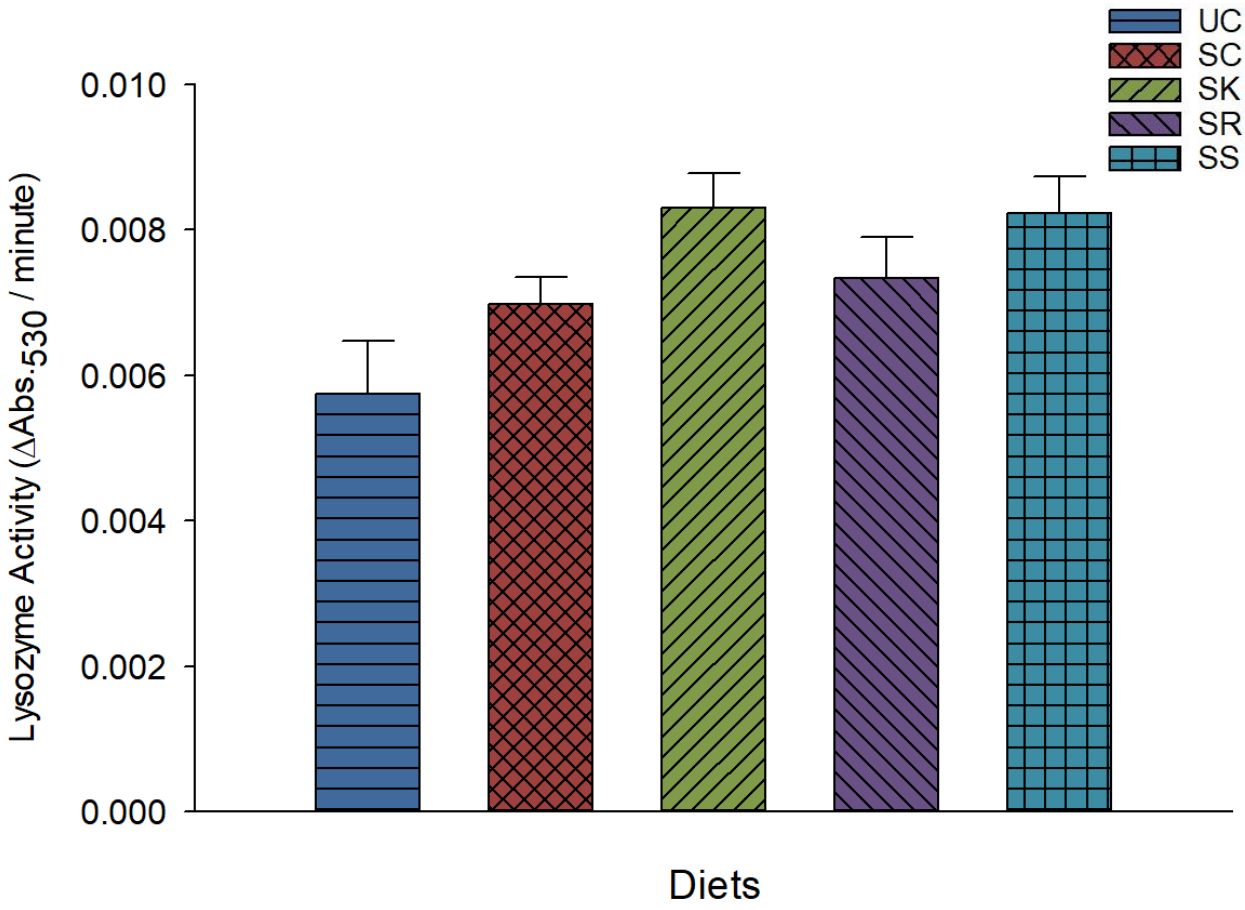

Figure 22: Overall main effect of diets on lysozyme activity. UC = unstressed fish fed commercial feed, SC = stressed fish fed commercial feed, SK = stressed fish fed kudzu (*Pueraria lobate*)-supplemented feed, SR = stressed fish fed red clover (*Trifolium pretense*)-supplemented feed, SS = stressed fish fed soybean (*Glycine max*)-supplemented feed. Bars represent mean + SEM.

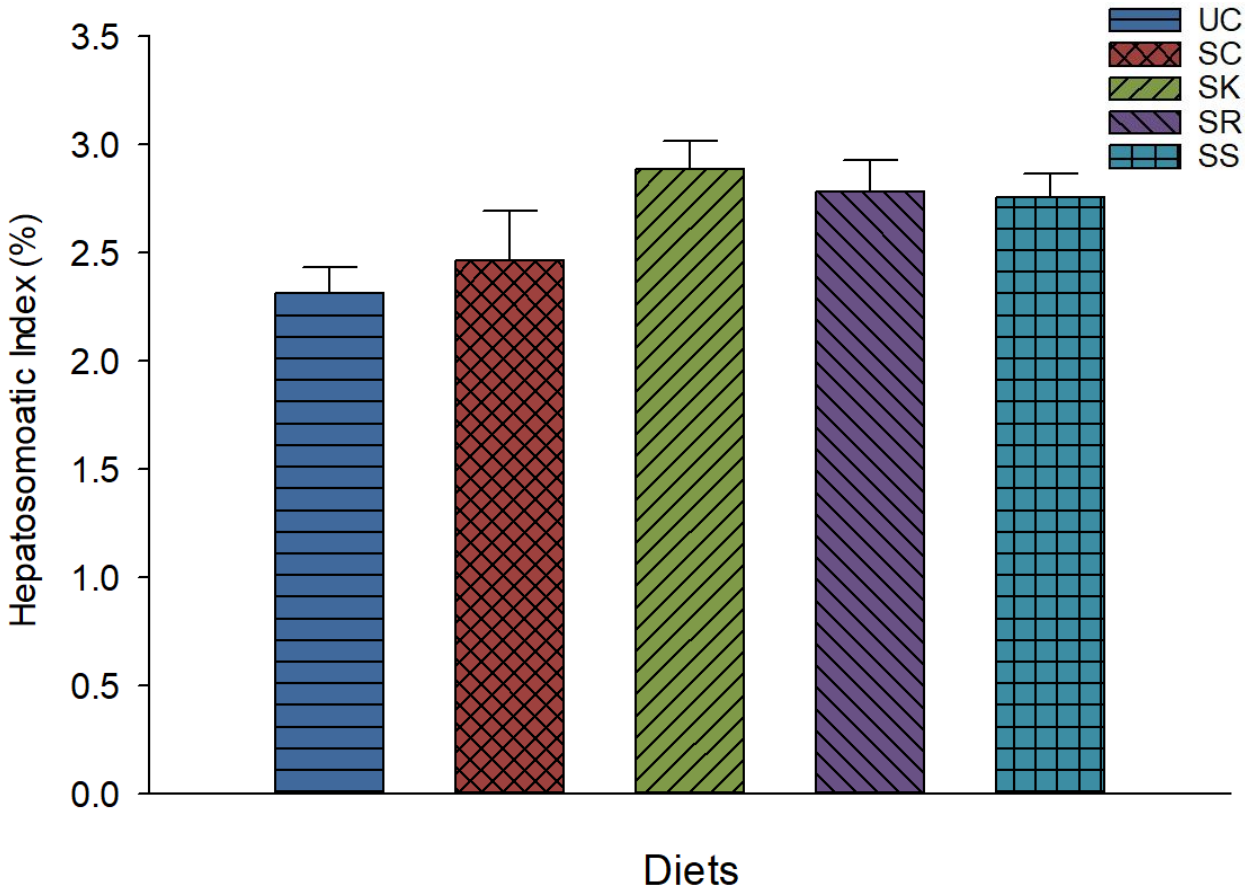

Figure 23: Overall main effect of diets on hepatosomatic index. UC = unstressed fish fed commercial feed, SC = stressed fish fed commercial feed, SK = stressed fish fed kudzu (*Pueraria lobate*)-supplemented feed, SR = stressed fish fed red clover (*Trifolium pretense*)-supplemented feed, SS = stressed fish fed soybean (*Glycine max*)-supplemented feed. Bars represent mean + SEM.

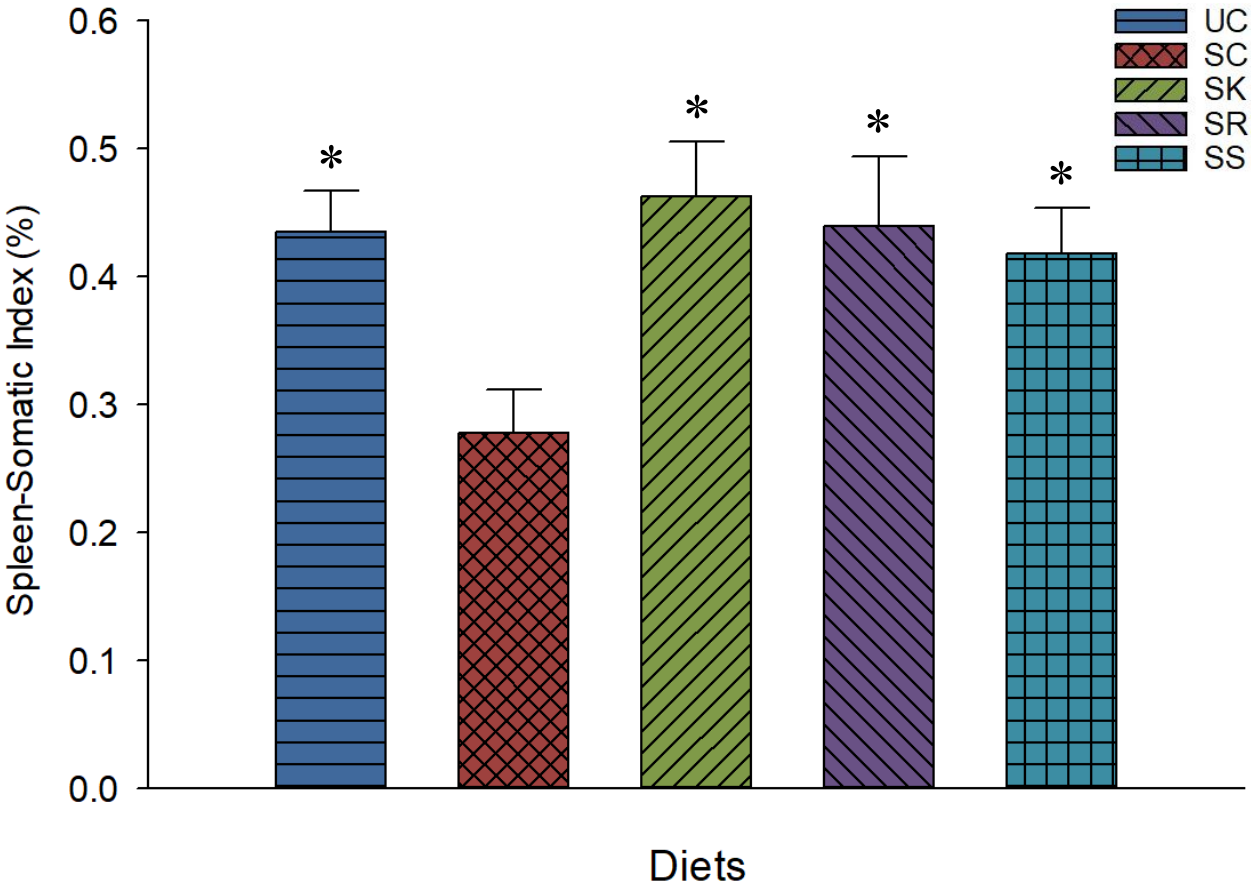

Figure 24: Overall main effect of diets on spleen-somatic index. UC = unstressed fish fed commercial feed, SC = stressed fish fed commercial feed, SK = stressed fish fed kudzu (*Pueraria lobate*)-supplemented feed, SR = stressed fish fed red clover (*Trifolium pretense*)-supplemented feed, SS = stressed fish fed soybean (*Glycine max*)-supplemented feed. Bars represent mean + SEM; * = $p < .05$ compared to SC.

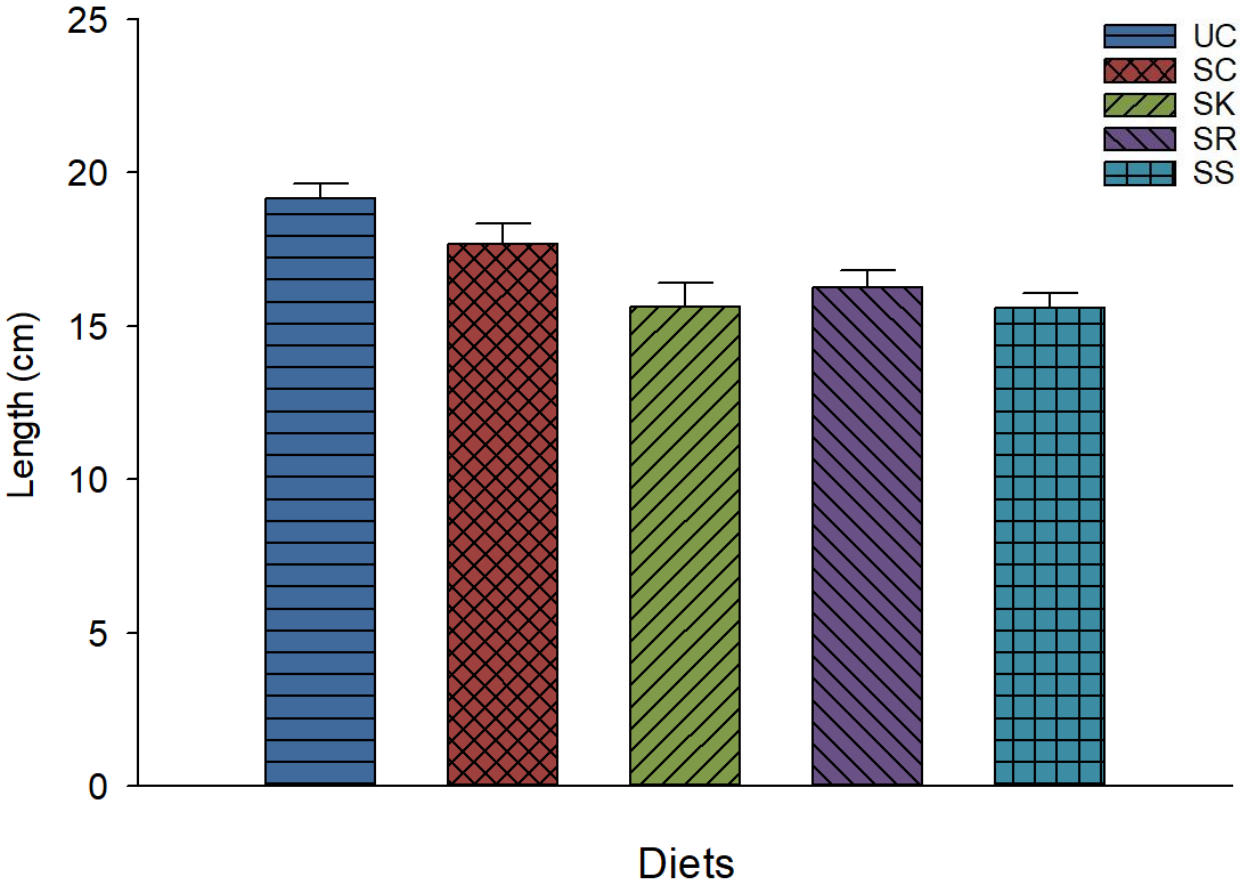

Figure 25: Effect of diets on terminal (day 42) length. UC = unstressed fish fed commercial feed, SC = stressed fish fed commercial feed, SK = stressed fish fed kudzu (*Pueraria lobate*)-supplemented feed, SR = stressed fish fed red clover (*Trifolium pretense*)-supplemented feed, SS = stressed fish fed soybean (*Glycine max*)-supplemented feed. Bars represent mean + SEM.

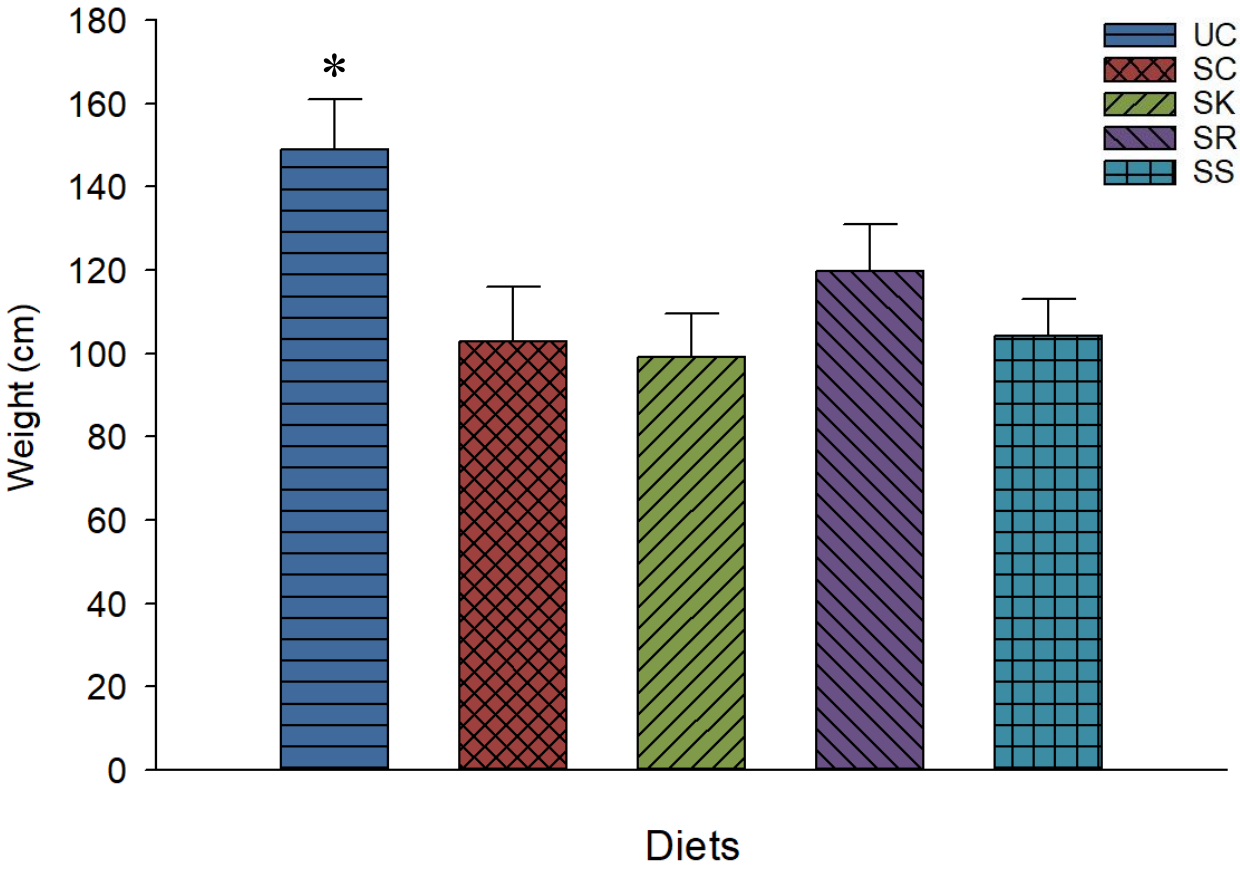

Figure 26: Effect of diets on terminal (day 42) weight. UC = unstressed fish fed commercial feed, SC = stressed fish fed commercial feed, SK = stressed fish fed kudzu (*Pueraria lobate*)-supplemented feed, SR = stressed fish fed red clover (*Trifolium pretense*)-supplemented feed, SS = stressed fish fed soybean (*Glycine max*)-supplemented feed.
Bars represent mean + SEM; * = $p < .05$ compared to SC.

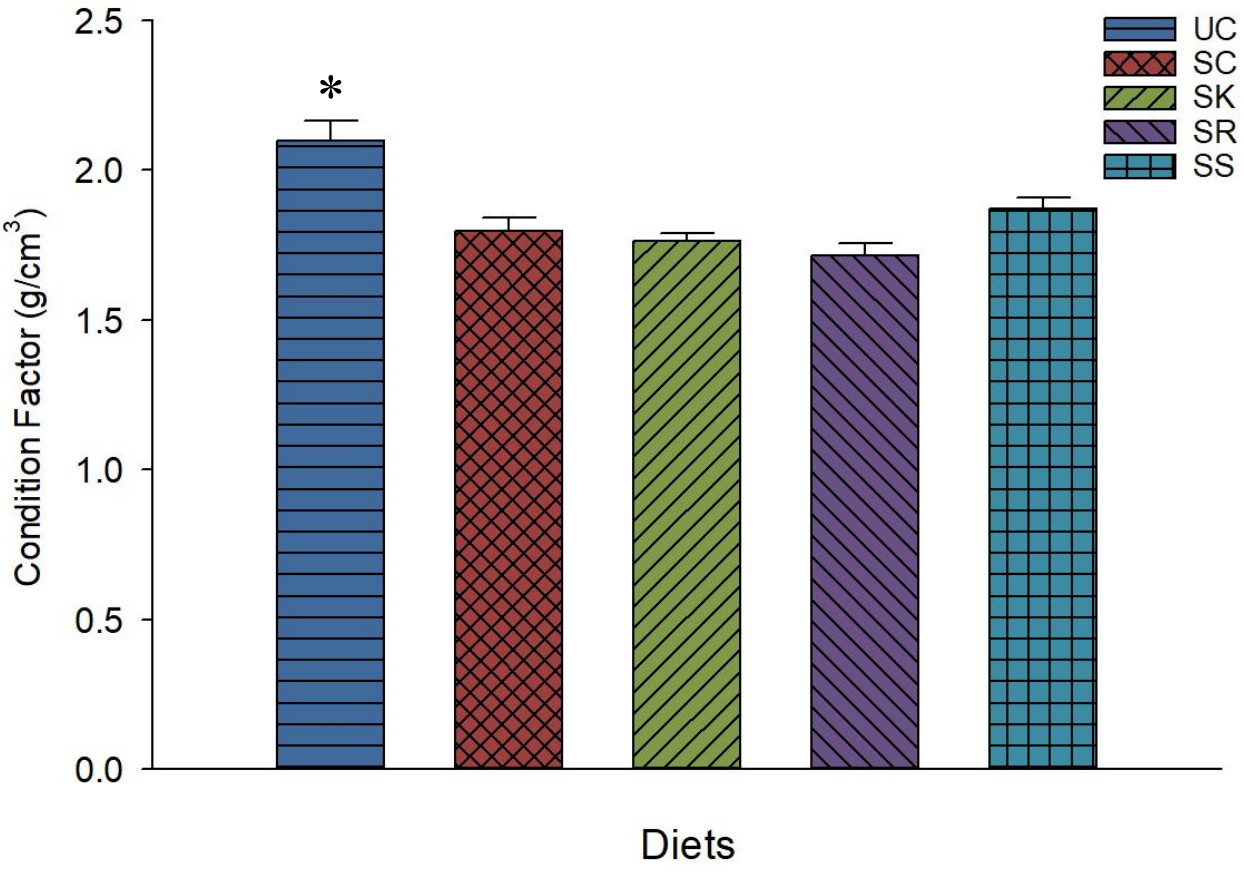

Figure 27: Effect of diets on terminal (day 42) condition factor. UC = unstressed fish fed commercial feed, SC = stressed fish fed commercial feed, SK = stressed fish fed kudzu (*Pueraria lobate*)-supplemented feed, SR = stressed fish fed red clover (*Trifolium pretense*)-supplemented feed, SS = stressed fish fed soybean (*Glycine max*)-supplemented feed. Bars represent mean + SEM; * = $p < .05$ compared to SC.

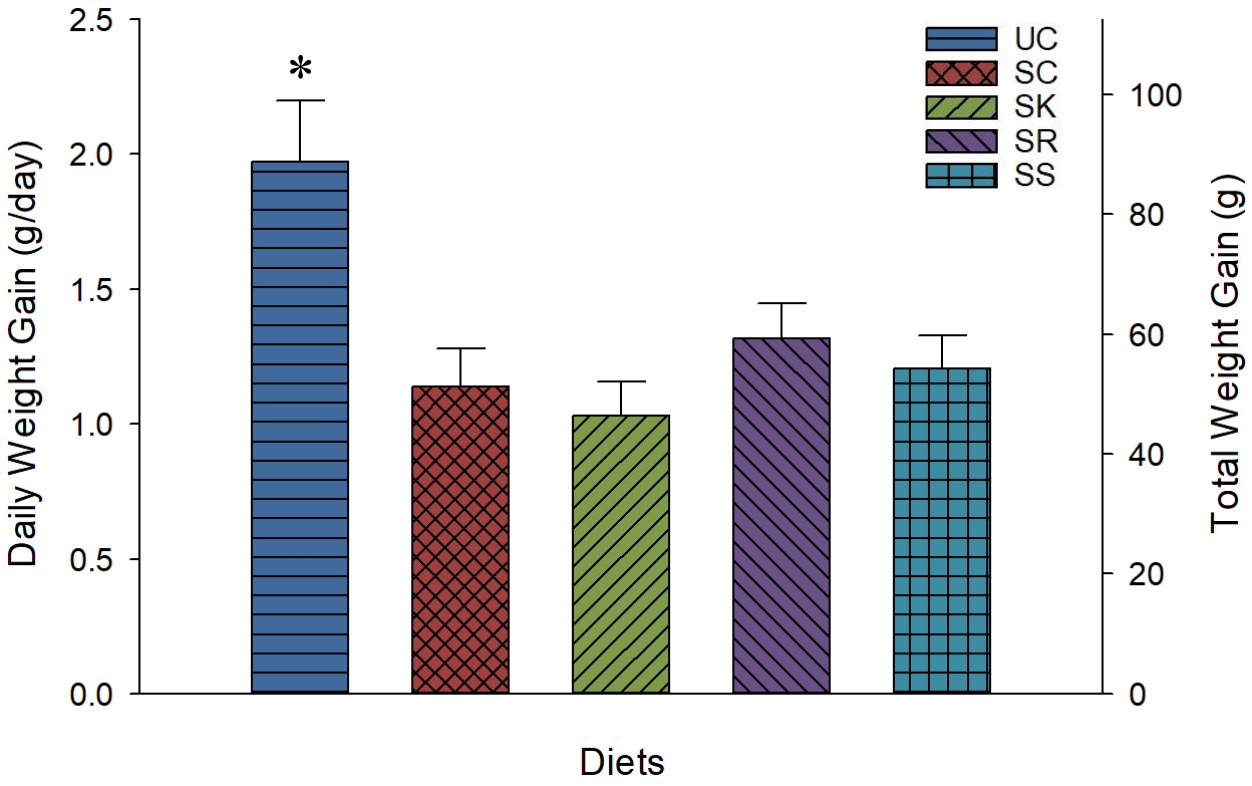

Figure 28: Effect of diets on daily and total weight gain over 42 days. UC = unstressed fish fed commercial feed, SC = stressed fish fed commercial feed, SK = stressed fish fed kudzu (*Pueraria lobate*)-supplemented feed, SR = stressed fish fed red clover (*Trifolium pretense*)-supplemented feed, SS = stressed fish fed soybean (*Glycine max*)-supplemented feed. Bars represent mean + SEM; * = $p < .05$ compared to SC.

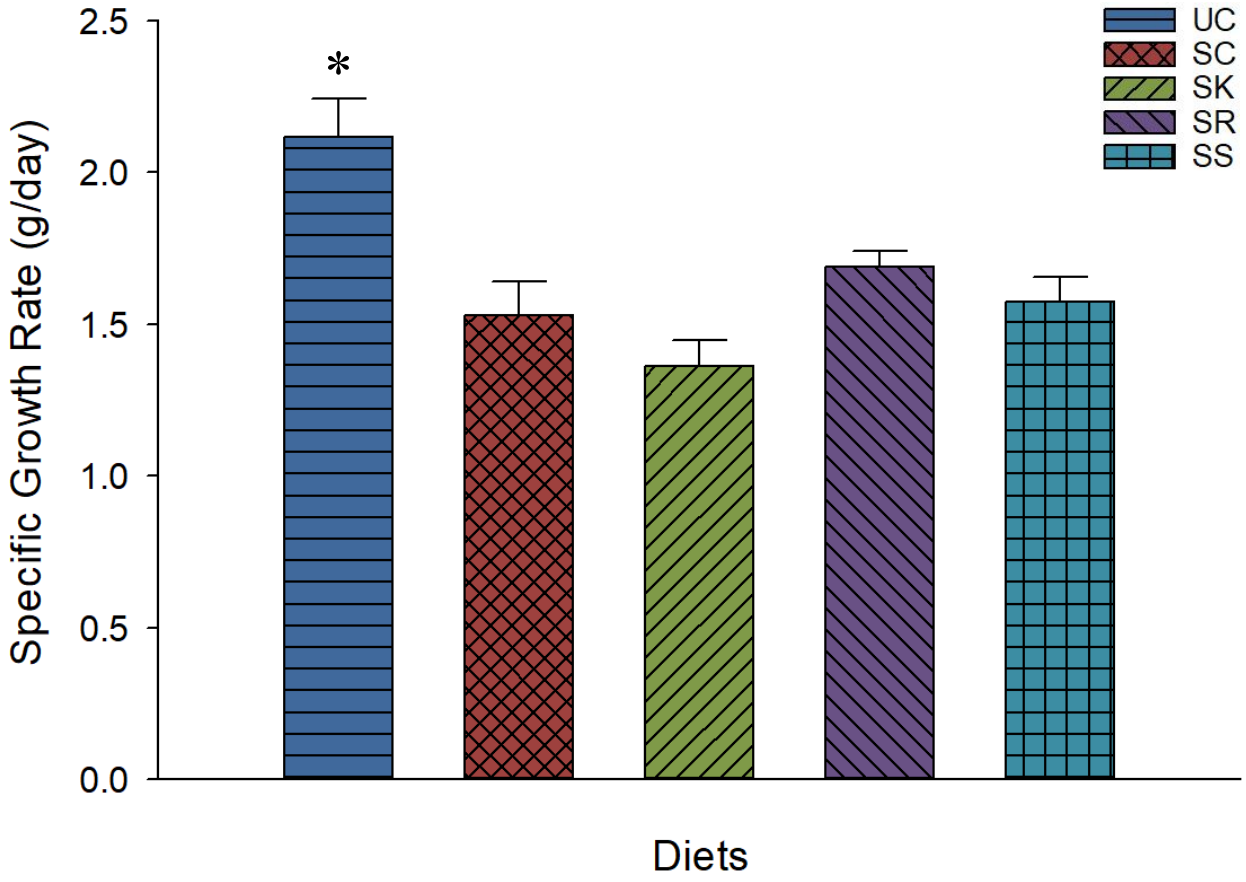

Figure 29: Effect of diets on specific growth rate over 42 days. UC = unstressed fish fed commercial feed, SC = stressed fish fed commercial feed, SK = stressed fish fed kudzu (*Pueraria lobate*)-supplemented feed, SR = stressed fish fed red clover (*Trifolium pretense*)-supplemented feed, SS = stressed fish fed soybean (*Glycine max*)-supplemented feed. Bars represent mean + SEM; * = $p < .05$ compared to SC.

Table 11: Effects of diets on B_Δ, W_Δ, SGR, FI, FCR, and PER over 6 weeks. UC = unstressed fish fed commercial feed, SC = stressed fish fed commercial feed, SK = stressed fish fed kudzu-supplemented feed, SR = stressed fish fed red clover-supplemented feed, SS = stressed fish fed soybean-supplemented feed. Values represent mean ± SEM. * = $p < .05$ compared to SC.

Growth Parameters	**UC**	**SC**	**SK**	**SR**	**SS**
Biomass (g)					
B_i	449.8	433.2	430.4	431.0	428.7
B_f	1115.6	815.7	776.8	874.2	834.4
Daily B_Δ	15.95	9.11	8.25	10.55	9.66
Total B_Δ	665.8	382.5	346.4	443.2	405.7
Weight (g)					
W_i	54.8 ± 2.7	54.2 ± 6.4	53.8 ± 4.0	57.3 ± 3.7	53.6 ± 4.1
W_f	149.0 ± 10.4 *	102.9 ± 12.3	104.5 ± 6.1	119.8 ± 8.8	104.3 ± 8.7
Daily W_Δ	1.97 ± 0.22 *	1.14 ± 0.14	1.03 ± 0.13	1.32 ± 0.13	1.21 ± 0.12
Total W_Δ	83.2 ± 9.2 *	47.8 ± 6.05	43.3 ± 5.4	55.4 ± 5.3	50.7 ± 5.2
SGR (g)	2.12 ± 0.12 *	1.53 ± 0.11	1.36 ± 0.09	1.69 ± 0.05	1.57 ± 0.08
FI (g)					
Daily FI	21.4	18.1	16.9	17.7	17.5
Total FI	900.7	759.6	707.9	744.6	734.7
FCR	1.35	1.99	2.04	1.68	1.81
PER	1.48	1.01	0.98	1.19	1.10

Discussion

All of the stressed groups showed a nonsignificant overall decrease in cortisol over the 10-day study. While fish fed red clover extract-supplemented feed showed comparable serum cortisol concentrations to the stress control fish, the kudzu and soy extract diets showed even lower concentrations of serum cortisol than the stress control, suggesting the addition of these isoflavones may cause additional stress beyond the effects of hydrocortisone added to the diets, or they lower cortisol concentration by some other mechanism. This decrease in serum cortisol concentration for stressed fish is most likely due to the addition of hydrocortisone to the feed for our stressed treatments, resulting in changes to the negative feedback mechanism that regulates cortisol. Repeated chronic stressors, such as the daily consumption of hydrocortisone, affects the feedback mechanism of the HPI axis, resulting in lower serum cortisol concentrations (Barton, 2002; Barton et al., 1987; Madaro et al., 2015). This is achieved through downregulation of cortisol receptors (Pottinger, 1990; Barton et al., 1987) and increased metabolism of cortisol (Redding et al., 1984).

No major differences in blood glucose concentration were detected between the unstressed and stressed controls used in this study. There did appear to be a slight overall increase in blood glucose concentration for all of the crude isoflavone extract-supplemented feed, with kudzu extract-supplemented feed showing the greatest increase in blood glucose. These differences appear to be negligible though. Meanwhile, there was an overall increase in hepatosomatic index associated with crude isoflavone supplementation, above that associated with stress alone. The liver is an important organ for homeostasis by storing energy in the form of glycogen (Wendelaar Bonga, 1997). This increase in liver size, while having minimal to no effect on blood glucose, would suggest that crude isoflavone extracts may improve energy reserves.

Stress resulted in a small overall decrease in hematocrit, or the percentage of red blood cells in the blood. Red clover and soybean extract-supplemented feed showed similar results for hematocrit as the stress fish fed commercial feed, but kudzu extract-supplemented feed showed an overall increase in hematocrit, above unstressed fish fed commercial feed. Evidence suggests that puerarin, the main component in kudzu, has hemolytic effects (Yue et al., 2008), although these findings have not been replicated in fish. The fish may be upregulating the production of

red blood cells to counteract the hemolytic effect, which would result in a greater hematocrit. Stress is known to cause increases in hematocrit due to the release of additional red blood cells in an attempt to maintain homeostasis through increased metabolism (Wendelaar Bonga, 1997). Compounded with the finding that kudzu extract supplementation also caused a decrease in cortisol, this would suggest that kudzu extract may produce negative side effects that result in increased stress.

Stress did not affect plasma protein concentrations, an indicator of innate immunity (Dawood et al., 2017; Gerwick et al., 2002), during acute stress, but as stress accumulated, there was an increase in protein concentration. Stressed fish fed crude isoflavone extract-supplemented feed showed increased plasma protein concentrations throughout the duration of the study, with this increase becoming more pronounced as stress continued for soybean extract-supplemented feed. Soybean isoflavones have previously demonstrated the capacity to increase serum protein concentration under stress (Zhou et al., 2015b). Under stress, soybean isoflavones were shown to upregulate the concentration of various proteins associated with innate immunity, such as catalase, superoxide dismutase, lysozyme, and different heat shock proteins (Zhou et al., 2015b; Yang 2019). This supports our finding of increased lysozyme activity associated with soybean extract supplementation. Soybean extract has also demonstrated the ability to increase lysozyme activity under normal, unstressed conditions (Zhou et al., 2015a). Soybean protein concentrate has also been shown to increase plasma protein concentration in fish (Rumsey et al., 1994). While quantification of isoflavones was not determined in the Rumsey et al. (1994) study, soybean products consistently show substantial concentrations of isoflavones (Chen and Wei, 2008). These findings support the findings of this study, suggesting soybean isoflavones have a potent impact on plasma protein concentration. It appears these upregulated proteins may help modulate some of the negative consequences of stress, such as the effect of stress on protein misfolding, as seen in the increase in heat shock proteins (Zhou et al., 2015b).

Stress caused an overall decrease in spleen-somatic index, but the addition of crude isoflavone extracts produced significant increases in relative spleen size. Spleen size has demonstrated a predictive ability in determining disease resistance, with larger spleens being associated with increased resistance (Hadidi et al., 2008). Stress is known to decrease spleen size, possibly due to

the effects of stress on lymphocyte apoptosis (Saha et al., 2003). The addition of crude isoflavone extracts demonstrated the ability to modulate the negative consequence of stress on spleen size. Evidence suggests that puerarin, the main isoflavone of the crude kudzu extract, stimulates an increase spleen size in both stressed and unstressed fish (Hossain et al., 2013).

In terms of growth, there were many clear differences between stressed and unstressed fish regarding the physical composition of the fish. Stressed showed decreases in terminal weight and length, contributing to a poorer condition factor. Crude isoflavone extract-supplemented feed appeared to decrease length beyond that of stress, while also having greater (in the case of red clover extract) or comparable (in the cases of kudzu and genistein extracts) weight as growth. One would expect this to improve condition factor, but the crude isoflavone extracts did not appear to cause a considerable change. The negative consequences of stress are also apparent when looking growth over the six-week period. Stress resulted in lower gains in biomass and weight, as well as a lower specific growth rate. Again, red clover extract-supplemented feed appeared to improve growth over this same period. Previous studies have shown similar results regarding the effects of red clover extract on growth. Red clover extract supplementation has previously increased weight gain and specific growth rate in fish (Turan and Akyurt, 2005). Soybean extract appeared to have little to no effect on overall growth, while kudzu appeared to decrease these growth rates. The use of daidzein and genistein in combination has also shown positive effects on growth in fish (Bagheri et al., 2014). Both the red clover and soybean extracts used in this study contain daidzein and genistein, suggesting the ratio between these isoflavones may be an important consideration. Red clover extract contains daidzein and genistein in relatively equal proportions, while soybean extract contains a higher concentration of daidzein compared to genistein. Positive growth was associated with these isoflavones being roughly proportional (Bagheri et al., 2014), as seen in the red clover extract used in this study.

These changes in growth caused by stress and crude isoflavone extract supplementation can be better understood by taking into consideration feed intake. Stress caused an overall decrease in feed intake while exhibiting higher costs in production as shown by the increase in feed conversion and the decrease in protein efficiency ratios. Kudzu extract-supplemented feed resulted in lower feed intake beyond that of stress alone, resulting in comparable feed conversion

and protein efficiency. Red clover and soybean extract supplementation appeared to lower feed intake, explaining the relative increases in feed conversion and a decreased protein efficiency. The effect of red clover and red clover extract on reduced feed intake in fish has been previously demonstrated (Turan, 2006; Turan and Akyurt, 2005).

Little information is available on the effects of kudzu extract affecting growth, let alone in fish. This study is a step in better understanding the effects of kudzu extract on growth under stress. There is conflicting information regarding the effects of soybean isoflavones on growth. Evidence suggests that soybean isoflavones either have no impact or inhibition on growth, depending on the concentration of soybean isoflavones added to the diet (Mai et al., 2012). While there were no differences in feed intake associated with soybean isoflavone use in the diet, there was a significant decrease in feed conversion at concentrations greater than what was used in this study (Mai et al., 2012). A recent study suggests that soybean isoflavones may improve growth, but again, is dependent on concentration (Yang, 2019). At low concentrations, soybean isoflavones greatly improved weight gain, specific growth rate, feed intake, and feed conversion, but these trends were reversed at greater concentrations of soybean isoflavones (Yang, 2019). This effect may be explained by the effects of soybean isoflavones on apoptosis in muscle cells. Soybean isoflavones were shown to reduce DNA fragmentation and apoptosis in myocytes at lower concentrations, resulting in greater muscle mass, but increased DNA fragmentation and apoptosis at greater concentrations (Yang, 2019). These findings strongly suggest that concentration of isoflavones, both overall and in conjugation with each other, is an important factor that needs to be considered.

Conclusion

Stress causes profound negative consequences on growth and immunity in fish. These are expected to be exacerbated as aquaculture continues to grow. The use of isoflavones in the diets show the potential to minimize some of these negative side effects to stress. Stress caused a decrease in cortisol due to the effects on hydrocortisone added to the feed affecting the HPI axis negative feedback mechanism. Red clover extract did not appear to improve cortisol, but the addition of kudzu and soybean extracts to the feed may further lower cortisol levels. It appears that crude isoflavone extracts may also increase energy reserves in Nile tilapia, as shown by an

increase in liver size while having minimal effects on blood glucose. These isoflavone extracts also appear to promote innate and adaptive immunity, as seen by the overall increase in plasma protein, lysozyme activity, and spleen size. Red clover extract supplemented to the feed appears to increase overall growth of Nile tilapia, while kudzu and soybean extracts showed minimal effects on growth. Of the three crude isoflavone extracts, red clover extract supplementation produced the best results in modulating stress, thereby improving immunity and growth. While more research is needed, the addition of red clover extract to the diet shows promising potentials in bettering the welfare of fish.

GENERAL DISCUSSION

Two studies were used to examine the effects of isoflavones on immunity and growth in stressed Nile tilapia. The first study focused on the use of pure isoflavone isolates, while the second examined the effects of crude isoflavone extracts. Focusing on the positive and negative stress controls used in this study, there appear to be some substantial differences. In the first study, there was a significant decrease in cortisol caused by the addition of hydrocortisone in the feed. While there was also a decrease in the second study, this decrease was not significant. The overall serum cortisol levels in the second study were lower than those in the first study, indicating the presence of additional stress. Stocking density is an important factor in rearing fish such as Nile tilapia, with aggression increasing as stocking density increases (Hecht and Appelbaum, 1988; Azaza et al., 2013). Aggression may be particularly impactful at lower densities though (Suresh and Lin, 1992), with greater aggression being correlated with an increase in stress (Barreto et al., 2009). In larger tanks with greater stocking density, as seen in the first study, Nile tilapia are able to spread the aggression throughout the population. In smaller setups, such as in the second study with only eight fish, aggression is not able to be dispersed throughout the population. This leads to a clear hierarchy, which has negative consequences on the fish (Harwood et al., 2003) in the form of increased stress. In the first study, there were clear significant differences between stressed and unstressed controls for many of our physiological parameters. These same differences were not always detected in the second study. Alternatively, statistical differences between stressed and unstressed fish regarding the growth parameters were consistently detected in both studies. Between the first and second studies, stressed controls showed very similar growth factors, such as the similar feed conversion and protein efficiency ratios seen between the two studies. Conversely, there was decreased feed conversion ratio and an increased protein efficiency ratio for the control group in the second study compared to the first. This would indicate the presence of additional stress (Wendelaar Bonga, 1997). Even though this confound should be considered with any comparisons between the two studies discussed hereafter, it would not invalidate the findings of the second study. Since all Nile tilapia were reared in the same conditions for the second study, the effect of low density on stress would be the same across all diets.

Under stress, blood glucose concentrations increase and red blood cells are released to meet increased energy demands to evade the noxious stimuli (Barton et al., 1987). The addition of cortisol to induce stress did not produce the expected elevation in blood glucose. While there was an apparent increase in hematocrit for the first study, no significant differences were detected between stressed and unstressed fish for the second study. An overall decrease in hepatosomatic index was also expected, as energy reserves are in the liver are utilized to help meet these increased energy demands (Barton et al., 1987). Again, neither study showed significant differences between stressed and unstressed controls for this physiological parameter. Plasma protein was shown to be susceptible to cortisol administration, consistently showing increased plasma protein concentrations. Increases in plasma protein concentrations were expected as certain proteins are upregulated to deal with the negative consequences of stress (Barton et al., 1987). Isoflavone supplementation showed a myriad of effects on plasma protein concentration, both across diets and at different durations of stress.

Across both studies, there is a clear effect of hydrocortisone administration on physiological changes, including decreased immunity and decreased growth, as energy is diverted from these functions to deal with the disruption of homeostasis induced by stress (Barton et al., 1987). Both pure isoflavone isolates and crude isoflavone extracts show the potential to alleviate these negative side effects of stress. There were numerous difference and similarities between the two supplements, even though the crude isoflavone extracts contained one or the other of the pure isoflavone isolates tested in the first study. This would suggest that there are interactive effects between isoflavones beyond those of the individual isoflavone isolate, as a few other studies have shown (Huang et al., 2016; Wu et al., 2019).

In the first study, pure puerarin isolate was shown to decrease hematocrit regardless of the duration of stress. In the second study, the use of crude kudzu extract, which mainly contains puerarin isoflavone, had the opposite effect and actually caused on overall increase in hematocrit. Kudzu extract also contained daidzin and daidzein, suggesting one of these two isoflavones (or possibly the combination of both) may produce these differing effects. Similar to isolated puerarin, isolated daidzein has shown the ability to decrease hematocrit in rats under conditions that would produce stress (Karale and Kamath, 2017). This would suggest that the

increase in hematocrit may be due to the effects of daidzin, or some unmeasured combined effect produced by the combination of diadzein, daidzin, and puerarin. Alternatively, there were similar trends between puerarin isolate and kudzu extract on the size of the liver in stressed Nile tilapia. Both puerarin isolate and kudzu extract were shown to increase hepatosomatic index under stress. Diadzein has been shown not to affect hepatosomatic index in fish (Hu, 2014), suggesting puerarin may be the cause of increased liver size.

Study one showed the effects of genistein isolate on plasma protein, with the addition resulting in an increase in plasma protein concentration. Soybean extract also showed the same trend. Both genistein isolate and soybean extract have been shown to increase superoxide dismutase and catalase in diabetic rats (Lee, 2006), both of which are found in the serum of fish (Feng et al., 2013). It is possible that the effects of genistein on plasma protein concentration explain the similar effects seen by soybean extract. The soybean extract used in this study also contained other isoflavones though, with diadzin being in the greatest abundance, followed by glycitin, genistin, daidzein, and glycitein, in that ordered. Genistein is actually the least abundant isoflavone found in this crude soybean extract. Glycitein, another isoflavone in low abundance in the soybean extract used in study two, has been shown to also increase superoxide dismutase activity when used as a feed additive, although there was no effect on catalase activity (Hu et al., 2015). The affinity of the isoflavones genistein and glycitein to plasma proteins may explain part of the mechanism behind these findings. Daidzin, genistin, and glycitin are glycosylated forms of daidzein, genistein, and glycitein, respectively, meaning they contain the addition of a carbohydrate to their molecular structure (Coward et al., 1993). Glycosylation (in the case of diadzin and genistin) decreased the affinity between the isoflavones and proteins in the serum when compared to their respective non-glycosylated molecules (Xiao et al., 2009). Even though genistein and glycitein are in relatively low abundance in the crude soybean extract, their molecular structure may allow them to produce greater effects than other, more abundant isoflavones, particularly on plasma protein concentration. The finding that isoflavones may promote immunity is also supported by the effects of the pure isoflavone isolates (study one) and crude isoflavone extracts (study two) on spleen size. These use of isoflavones, regardless of preparation, resulted in increased spleen-somatic index, which is predictive of increased immunity and disease resistance (Hadidi et al., 2008). This finding was repeatedly shown to be

significant, often producing effects that increased relative spleen size to that above the unstressed control. Puerarin was present in kudzu extract, while genistein was present in both red clover and soybean extract, indicating that these isoflavones may play a role in increasing spleen-somatic index. Taken together, these findings from the physiological parameters measured in both studies suggest a potent effect of isoflavones on reducing stress and improving immunity, although more research is needed to determine the individual effects of these isoflavones and their respective mechanisms of action.

The use of isoflavones generally improved the growth of Nile tilapia as well. In study one, the use of puerarin and genistein resulted in increased growth rates. Puerarin and genistein supplementation also resulted in a lower feed conversion ratio and increased protein efficiency ratio, while having minimal to no impact on feed intake compared to the stress control. Genistein was associated with a greater condition factor, which was also seen in fish fed soybean extract-supplemented feed. Similar growth findings with red clover extract were shown in the second study. The use of red clover extract-supplemented feed resulted in increased growth rate, while having minimal effects on feed intake, resulting in better feed conversion and protein efficiency. Red clover contains genistein is minimal amounts, meaning genistein may partially explain these effects. Genistein is also found in soybean extract, but soybean extract supplemented in the feed showed minimal improvements in growth. Since genistein has shown the ability to increase growth in study one as well as in other research (Ko et al., 1999), this leads to two testable (although not necessarily mutually exclusive) hypotheses to explain these different results for red clover and soybean extracts: 1) genistein is in too low of abundance to cause improvements in growth under stress, meaning a different isoflavone is responsible for this increased growth in red clover, or 2) the interaction between the isoflavones in soybean extract in some way minimize the individual effect of genistein on growth. Research suggests the second hypothesis is more likely. The use of daidzein (present in both red clover and soybean extract) was shown to not significantly affect growth in fish (Hu, 2014). A recent studying examining the effects of biochanin A (abundant isoflavone in red clover extract) and daidzein (relatively low abundance in both red clover and soybean extract) were shown to have no impacts on growth, feed intake, feed conversion, or protein efficiency either in fish (Fickler et al., 2019). This suggests that either formononetin in red clover extract is responsible for the increase in growth, or again, a possible

interaction that would require more research. Interactions between these isoflavones would also be expected based on the findings that puerarin increased growth while kudzu extract, which contains mainly puerarin, did not improve growth. Isoflavones as a whole are potentially growth modulators under stress, but more research is needed to determine the individual and combined effects of these isoflavones.

GENERAL CONCLUSION

Scientists are developing a better understanding of the widespread health benefits of isoflavones. While further research is needed, evidence suggests the addition of isoflavones to the feed of Nile tilapia will reduce stress, improve immunity, and promote growth. These pure isoflavone isolates have shown the potential to improve serum cortisol concentration, a strong indicator of stress in animals. These both pure isolates and crude extracts of isoflavones may also produce immune stimulating benefits, as seen in the increase of plasma protein concentration, lysozyme activity, and spleen-somatic index. Isoflavones, particularly pure isoflavone isolates, also appear to influence growth, improving overall growth rates while maintaining similar feed requirements, resulting in better conversion of feed into body weight and protein. There is still much to understand about these isoflavones. While research is ongoing into the individual effects of isoflavones on a variety of biologically important parameters, relatively little research exists that focuses on the combined effects of isoflavones. As aquaculture continues to grow, it is expected that the stressors will increase and the consequences of those stressors will increase alongside. Not only should the welfare of the animal be concerned, but reducing stress in the aquaculture environment is beneficial to all people. The use of isoflavones may be one tool to achieve this result. Reduction in stress through the use of isoflavones could lead to a decrease of antibiotics and chemicals in aquaculture, possibly reducing pressures on antibiotic resistance and the consumption of antibiotics as well as reducing pollutants in the environment. More research is need though to understand the direct effects of isoflavone supplementation on antibiotic resistance. Isoflavones show the potential to increase Nile tilapia production, which not only rewards industry, but helps supply the increasing global demands of high quality fish protein.

LIMITATIONS

There are four major limitations to this study. First, the presence of controls for the pure isoflavone and isoflavone extract treatment diets would have allowed for a much deeper understanding into the effects of isoflavones. Determining the effects on unstressed of these pure isoflavone isolates and crude isoflavone extracts would not only allow for additional comparisons, but would provide insights into the effects of these isoflavones in healthy Nile tilapia. While this limitation hindered the ability to further understand the mechanisms behind these isoflavones, it did not detract from the main objectives of this thesis, which was to determine the varying effects of isoflavones in *stressed* Nile tilapia. Second, although previously discussed in more detail, the consequences of increased aggression at such low densities was an underlying confound that should be considered for all comparisons between study one and study two. The benefit of rearing fish in smaller tanks at lower densities was the ability to statistically analyze weight gain and specific growth rate between diets without the use of tagging. Third, while terminal length, weight, and condition factor (weight gain and specific growth rate as well for the second study) was able to be statistically analyzed, much of the growth data is qualitative and would need to be reproduced to determine if these differences are statistically significant. Lastly, the dosage of isoflavones used to supplement the feed in this study was selected based off previous research showing success with isoflavone concentrations at this dosage. Dosages vary widely in the literature, showing varying effects at varying concentrations. Conclusions derived from these results should be understood in the context of the dosage, with the possibility that these effects may be enhanced or minimized at different dosages.

FUTURE DIRECTIONS

There are many directions in where this research could lead. First, these studies have demonstrated the importance of properly measuring and reporting the concentrations of isoflavones present in any diet supplementation. It is possible that many of the differing results seen in the literature are mainly due to differences in the concentrations. High performance liquid chromatography and other analytical tools to quantify the relative proportions of isoflavones in extracts and other isoflavone-containing products should be implemented. This would also allow for a better understanding of the combined effects of isoflavones, another area that requires much more research. Due to the limited information available about the combined effects of even two isoflavones, research should focus on the combined effects of the most abundant isoflavones, such as biochanin A, daidzein, genistein, and puerarin. When studying these combined effects, the use of graded levels of each isoflavone may provide a richer understanding. This would lead to future developments in determining the appropriate concentrations to use to promote the desired effects. While this research has examined some of the mechanisms behind the effects of isoflavones on stress, a deeper understanding is required and should be a major focus moving forward. The use of more advanced research techniques, including qPCR for gene transcription and western blot for protein production, would allow for a better understanding of the molecular messengers involved in these pathways. More research should also be dedicated to the cost-effectiveness of introducing isoflavones into the diet. As expected, pure isoflavones are extraordinarily more expensive than their crude extract counterparts. If crude extracts can produce the same, or even better, effects than the individual isoflavones, this would increase the likelihood that these isoflavones could be implemented in the diet on a large scale. And lastly, pilot studies using isoflavones in large-scale aquaculture operations are desperately needed.

IMPACT

Findings of this research could promote the overall welfare of fish by reducing stress, improving immunity, and boosting growth. The increase in growth associated with decreased stress would allow for increased production in aquaculture, allowing aquaculture to meet the growing demands for high quality protein and help feed many around the world. The reduction of stress could also lead to reduced uses of chemicals to treat fish affected by the consequences of stress. Less use of antibiotics in Eastern countries and elsewhere would lead to less selective pressure for antibiotic resistance and can lead to the decreased consumption of antibiotics by consumers. Fewer chemicals would also help protect the environment from aquaculture pollutants. This research could also lead to a better understanding of stress. Fish, being a vertebrate, share nearly identical mechanisms for stress compared to mammals. Stressors in the environment induce stress via the HPI axis in fish or the homologous HPA axis in mammals. Using fish to better understand the effects of isoflavones on stress could also lead for potential treatments using isoflavones in other animals, including humans.

REFERENCES

Azaza, M. S., Assad, A., Maghrbi, W., & El-Cafsi, M. (2013). The effects of rearing density on growth, size heterogeneity and inter-individual variation of feed intake in monosex male Nile tilapia *Oreochromis niloticus* L. *Animal: An International Journal of Animal Bioscience*, *7*(11), 1865-74.

Bagheri, T., Imanpoor, M. R., & Jafari, V. (2014). Effects of diets containing genistein and daidzein in a long-term study on sex steroid dynamics of goldfish (*Carassius auratus*). *Toxicology and Industrial Health*, *30*(2), 132-40.

Baras, E., Jacobs, B., & Melard, C. (2001). Effect of water temperature on survival, growth and phenotypic sex of mixed (XX-XY) progenies of Nile tilapia *Oreochromis niloticus*. *Aquaculture*, *192*(2-4), 187-199.

Barreto, R. E., Volpato, G. L., de Brito Faturi, C., Giaquinto, P. C., de Freitas, E. G., & de Castilho, M. F. (2009) Aggressive behaviour traits predict physiological stress responses in Nile tilapia (*Oreochromis niloticus*). *Marine and Freshwater Behaviour and Physiology*, *42*(2), 109-118.

Barton, B. A. (2002). Stress in fish: a diversity of responses with particular reference to changes in circulating corticosteroids. *Integrative and Comparative Biology*, *42*(3), 517-525.

Barton, B. A. & Iwama, G. K. (1991). Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. *Annual Review of Fish Diseases*, *1*, 3-26.

Barton, B., Schreck, C. B., & Barton, L. (1987). Effects of chronic cortisol administration and daily acute stress on growth, physiological conditions, and stress response in juvenile rainbow trout. *Diseases of Aquatic Organisms*, *2*, 173-185.

Basu, J., Nandi, J., & Bern, H. A. (1965). The homolog of the pituitary-adrenocortical axis in the teleost fish *Tilapia mossambica*. *Journal of Experimental Zoology*, *159*, 347-356

Bennetau-Pelissero, C., Breton, B., Bennetau, B., Corraze, G., Le Menn, F., Davail-Cuisset, B., Helou, C. & Kaushik, S.J. (2001) Effect of genistein-enriched diets on the endocrine process of gametogenesis and on reproduction efficiency of the rainbow trout *Oncorhynchus mykiss*. *General and Comparative Endocrinology*, *121*, 173– 187.

Blicharski, T. & Oniszczuk, A. (2017). Extraction methods for the isolation of isoflavonoids from plant material. *Open Chemistry*, *15*(1), 34-45.

Braithwaite, R. A., Carracosa, M. C., & McEvoy, L. A. (2006) Biofouling of salmon cage netting and the efficacy of a typical copper-based antifoulant. *Aquaculture*, *262*, 219-226

Brum, A., Pereira, S. A., Owatari, M. S., Chagas, E. C., Chaves, F. C. M., Mourino, J. L. P., & Martins, M. L. (2017). Effect of dietary essential oils of clove basil and ginger on Nile tilapia (*Oreochromis niloticus*) following challenge with *Streptococcus agalactiae*. *Aquaculture, 468*, 235-243.

Burdette, C. Q., & Marcus, R. K. (2013). Determination of isoflavone content in soy, red clover, and kudzu dietary supplement materials by liquid chromatography-particle beam/electron ionization mass spectrometry. *Journal of AOAC International, 96*(5), 925-932.

Burridge, L., Weis, J. S., Cabello, F., Pizarro, J., & Bostick, K. (2010). Chemical use in salmon aquaculture: A review of current practices and possible environmental effects. *Aquaculture, 306*(1-4), 7-23.

Butkute, B., Lemeziene, B., Dabkeviciene, G., Jakstas, V., Vilcinskas, E., & Janulis, V. (2014). Source of variation of isoflavone concentrations in perennial clover species. *Pharmacognosy Magazine, 10*(37), 181-188.

Caruso, G. (2016). Antibiotic resistance in fish farming environments: A global concern. *Journal of FisheriesSciences.com*, *10*(4), 9-13.

Cayman Scientific (2017). Cortisol ELISA Kit. Retrieved from https://www.caymanchem.com/pdfs/500360.pdf

Chen, T. R., & Wei, Q. K. (2008). Analysis of bioactive aglycone isoflavones in soybean and soybean products. *Nutrition & Food Science, 38*(6), 540-547

Coward, L., Barnes, N. C., Setchell, K. D. R., & Barnes, S. (1993). Genistein, daidzein, and their beta-glycoside conjugates: antitumor isoflavones in soybean foods from American and Asian diets. *Journal of Agriculture and Food Chemistry*, *41*(11), 1961-1967.

da Silva, R. L., Damasceno, F. M., Rocha, M. K. H. R., Sartori, M. M. P., Barros, M. M., & Pezzato, L. E. (2017). Replacement of soybean meal by peanut meal in diets for juvenile Nile tilapia, *Oreochromis niloticus*. *Latin American Journal of Aquatic Research*, *45*(5), 1044-1053.

Dawood, M. A., O., Koshio, S., Ishikawa, M., El-sabagh, M., Yokoyama, S., Wang, W. L., Yukun, Z., & Olivier, A. (2017). Physiological response, blood chemistry profile and mucus secretion of red sea bream (*Pagrus major*) fed diets supplemented with lactobacillus rhamnosus under low salinity stress. *Fish Physiology and Biochemistry*, *43*(1), 179-192.

Delmonte, P., Perry, J., & Rader, J. I. (2006). Determination of isoflavones in dietary supplements containing soy, red clover and kudzu: Extraction followed by basic or acid hydrolysis. *Journal of Chromatography.A, 1107*(1-2), 59-69.

Dhabhar, F. S. (2009). A hassle a day may keep the pathogens away: The fight-or-flight stress response and the augmentation of immune function. *Integrative and Comparative Biology, 49*(3), 215-236.

DiMaggio, M. A., Kenter, L. W., Breton, T. S., & Berlinsky, D. L. (2016). Effects of dietary genistein administration on growth, survival and sex determination in southern flounder, *Paralichthys lethostigma*. *Aquaculture Research, 47*(1), 82-90.

Ellis, A. E. (1990) Lysozyme activity. In: Stolen, T. C., Fletcher, P. D., Anderson, B. S., Roberson B. S., & Muiswinkel, W. B. Editors, Technique in Fish Immunology, SOS Publications, New Jersey, USA (1990), pp. 101–103

Ellis, T., Yildiz, H. Y., López-olmeda, J., Spedicato, M. T., Tort, L., Øverli, Ø., & Martins, C. I. (2012). Cortisol and finfish welfare. *Fish Physiology and Biochemistry*, *38*(1), 163-188.

Feng, L., Zhao, B., Chen, G., Jiang, W., Liu, Y., Jiang, J., Hu, K., Li, S., & Zhou, X. (2013). Effects of dietary histidine on antioxidant capacity in juvenile jian carp (*Cyprinus carpio* var. jian). *Fish Physiology and Biochemistry, 39*(3), 559-71.

Fickler, A., ×, S. S., Rimbach, G., & Schulz, C. (2019). Screening dietary biochanin A, daidzein, equol and genistein for their potential to increase DHA biosynthesis in rainbow trout (*Oncorhynchus mykiss*). *PLoS One*, *14*(1), 1-19.

Food and Agriculture Organization of the United Nations (2018). FAO yearbook. *Fishery and Aquaculture Statistics 2016*. Rome

Ganai, A. A., & Farooqi, H. (2015). Bioactivity of genistein: a review of in vitro and in vivo studies. *Biomedicine & Pharmacotherapy*, *76*, 30-38.

GeneCards database. GeneCards database [http://www.genecards.org], Assessed March 31, 2019.

Gerwick, L., Steinhauer, R., Lapatra, S., Sandell, T., Ortuno, J., Hajiseyedjavadi, N., & Bayne, C. J. (2002). The acute phase response of rainbow trout (*Oncorhynchus mykiss*) plasma proteins to viral, bacterial and fungal inflammatory agents. *Fish & Shellfish Immunology, 12*(3), 229-242.

Harvey, B. (2017). Regional review on status and trends in aquaculture development in North America – 2015. *FAO Fisheries and Aquaculture Circular 1135*(2), 1-30

Harwood, A. J., Armstrong J. D., Metcalfe, N. B., & Griffiths, S. W. (2003) Does dominance status correlate with growth in wild stream-dwelling Atlantic salmon (*Salmo salar*)? *Behavioral Ecology*, *14*, 902–908.

Hossain, M. R., Blumenthal, E., & Mustafa, A. (2013). Effects of puerarin on the reduction of glucose and promotion of overall health in acutely stressed chinook salmon, *Oncorhynchus tshawytscha* (Actinopterygii: Salmoniformes: Salmonidae). *Acta Ichthyologica Et Piscatoria, 43*(2), 85-93.

Hossain, M. R., & Mustafa, A. (2014). Puerarin promotes overall health through blood flow and immune response within chinook salmon (*Oncorhynchus tshawytscha*) fingerlings. *Journal of Applied Aquaculture, 26*(2), 119.

Hu, H. (2014). Effects of dietary daidzein on growth performance, activities of digestive enzymes,anti-oxidative ability and intestinal morphology in juvenile turbot (*Scophthalmus maximus* L.). *Journal of Fisheries of China/Shuichan Xuebao, 38*(9), 1503-1513.

Hu, Y. J., Gao, K. G., Zheng, C. T., Wu, Z. J., Yang, X. F., Wang, L., Ma, X. Y., Zhou, A. G., & Jiang, Z. J. (2015). Effect of dietary supplementation with glycitein during late pregnancy and lactation on antioxidative indices and performance of primiparous sows. *Journal of Animal Science, 93*(5), 2246-2254.

Huang, S., Su, S., Chang, J., Lin, H., Wu, W., Deng, J., & Huang, G. (2016). Antioxidants, anti-inflammatory, and antidiabetic effects of the aqueous extracts from *Glycine* species and its bioactive compounds. *Botanical Studies (Online), 57*(1), 1-11.

Hvistendahl, M. (2012). China takes aim at rampant antibiotic resistance. *Science*, *336*(6083), 795

International Food Policy Research Institute (2016). *Global Nutrition Report 2016: From Promise to Impact: Ending Malnutrition by 2030.* Washington, D.C.

Jaspe, C. J. & Caipang, C. M. A., 2011. Small-scale hatchery and larval rearing techniques for local strains of saline tolerant tilapia, *Oreochromis* spp. *ABAH Bioflux*, *3*, 71-77.

Jiang, R. W., Lau, K. M., Lam, H. M., Yam, W. S., Leung, L. K., Choi, K. L., Waye, M. M., Mak, T. C., Woo, K. S., & Fung, K. P. (2005). A comparative study on aqueous root extracts of *Pueraria thomsonii* and *Pueraria lobata* by antioxidant assay and HPLC fingerprint analysis. *Journal of Ethnopharmacology*, *96*(1-2), 133-138,

Karale, S., & Kamath, J. (2017). Effect of daidzein on cisplatin-induced hematotoxicity and hepatotoxicity in experimental rats. *Indian Journal of Pharmacology*, *49*(1), 49-54.

Klejdus, B., Mikelová, R., Petrlová, J., Poteseil, D., Adam, V., Stirborová, M., Hodek, P., Vacek, J., Kizek, R., & Vacek, J. (2005). Evaluation of isoflavone aglycon and glycoside distribution in soy plants and soybeans by fast column high-performance liquid chromatography coupled with a diode-array detector. *Journal of Agriculture and Food Chemistry*, *53*, 5848-5852.

Klonoff, D. C., Parks, J. L., Kovatchev, B. P., Kerr, D., Bevier, W. C., Brazg, R. L., Christiansen, M., Bailey, T. S., Nichols, J. H., & Kohn, M. A. (2018). Investigation of the accuracy of 18 marketed blood glucose monitors. *Diabetes Care*, *41*(8), 1681-1688.

Ko, K., Malison, J.A. & Reed, J.D. (1999) Effect of genistein on the growth and reproductive function of male and female yellow perch *Perca flavescens*. *Journal of the World Aquaculture Society*, *30*, 73– 79.

Hadidi, S., Glenney, G. W., Welch, T. J., Silverstein, J. T., & Wiens, G. D. (2008). Spleen size predicts resistance of rainbow trout to flavobacterium psychrophilum challenge. *Journal of Immunology, 180*(6), 4156-4165.

Hecht, T., & Appelbaum, S. (1988). Observations on intraspecific aggression and coeval sibling cannibalism by larval and juvenile *Clarias gariepinus* (clariidae: Pisces) under controlled conditions. *Journal of Zoology*, *214*(1), 21-44.

Lee, J. (2006). Effects of soy protein and genistein on blood glucose, antioxidant enzyme activities, and lipid profile in streptozotocin-induced diabetic rats. *Life Sciences, 79*(16), 1578-1584.

Little, D. C., Newton, R. W., & Beveridge, M. C. M. (2016). Aquaculture: a rapidly growing and significant source of sustainable food? Status, transitions and potential. *Proceedings of the Nutrition Society*, *75*, 274-286.

Liu, X., Steel, J. C., & Meng, X. Z. (2017). Usage, residue, and human health risk of antibiotics in Chinese aquaculture: a review. *Environmental Pollution*, *223*, 161-169.

Madaro, A., Olsen, R. E., Kristiansen, T. S., Ebbesson, L. O. E., Nilsen, T. O., Flik, G., & Gorissen, M. (2015). Stress in atlantic salmon: Response to unpredictable chronic stress. *The Journal of Experimental Biology, 218*, 2538-2550.

Mai, K., Zhang, Y., Chen, W., Xu, W., Ai, Q., & Zhang, W. (2012) Effects of dietary soy isoflavones on feed intake, growth performance and digestibility in juvenile Japanese flounder (*Paralichthys olivaceus*). *Journal of Ocean University of China* **11**, 511– 516.

Malik, A., Abbas, G., Ghaffar, A., Ferrando, S., Gallus, L., & Shah, S. S. A. (2018). Effect of different salinity level on breeding, fertilization, hatching and survival of Nile tilapia, *Oreochromis niloticus* (Linnaeus, 1758) in captivity. *Pakistan Journal of Zoology*, *50*(2), 539-547.

Mukne, A., Viswanathan, V., & Phadatare, A. (2011). Structure pre-requisites for isoflavones as effective antibacterial agents. *Pharmacognosy Reviews, 5*(9), 13-18.

Nardocci, G., Navarro, C., Cortés, P.,P., Imarai, M., Montoya, M., Valenzuela, B., Jara, P., Acuna-Castillo, C., & Fernández, R. (2014). Neuroendocrine mechanisms for immune system regulation during stress in fish. *Fish & Shellfish Immunology*, *40*(2), 531-538.

Olsvik, P. A., Skjærven, K. H., & Søfteland, L. (2017). Metabolic signatures of bisphenol A and genistein in Atlantic salmon liver cells. *Chemosphere, 189*, 730-743.

Panigrahi, A., Kiron, V., Satoh, S., & Watanabe, T. (2010). Probiotic bacteria lactobacillus rhamnosus influences the blood profile in rainbow trout *Oncorhynchus mykiss* (Walbaum). *Fish Physiology and Biochemistry*, *36*(4), 969-77.

Pilsáková, L., Riecanský, I., & Jagla, F. (2010). The physiological actions of isoflavone phytoestrogens. *Physiological Research, 59*(5), 651-64.

Pollack, S. J., Ottinger, M. A., Sullivan, C. V. & Woods, L. C. (2003) The effects of the soy isoflavone genistein on the reproductive development of striped bass. *North American Journal of Aquaculture*, *65*, 226– 234.

Pool-Zobel, B. L., Adlercreutz, H., Glei, M., Liegibel, U. M., Sittlington, J., Rowland, I., Wahala, K., & Rechkemmer, G. (2000). Isoflavones and lignans have different potentials to modulate oxidative genetic damage in human colon cells. *Carcinogenesis*, *21*, 1247-1252.

Pottinger, T. G. (1990). The effect of stress and exogenous cortisol on receptor-like binding of cortisol in the liver of rainbow trout, *Oncorhynchus mykiss*. *General and Comparative Endocrinology*, *78*(2), 194-203.

Rakocy, J. E. (2019). Cultured aquatic species information programme. *Oreochromis niloticus*. *In: FAO Fisheries and Aquaculture Department*. Rome.

Redding, J. M., Patiño, R., & Schreck, C. B. (1984). Clearance of corticosteroids in yearling coho salmon, *Oncorhynchus kisutch*, in fresh water and seawater and after stress. *General and Comparative Endocrinology, 54*(3), 433-443.

Reppert, A., Yousef, G. G., Rogers, R. B., & Lila, M. A. (2008). Isolation of radiolabeled isoflavones from kudzu (*Pueraria lobata*) root cultures. *Journal of Agricultural and Food Chemistry, 56*(17), 7860-7865.

Roosta, Z., Hajimoradloo, A., Ghorbani, R., & Hoseinifar, S. H. (2014). The effects of dietary vitamin C on mucosal immune responses and growth performance in Caspian roach (*Rutilus rutilus caspicus*) fry. *Fish Physiology and Biochemistry, 40*(5), 1601-1607.

Ruiz-Larrea, M. B., Mohan, A. R., Paganga, G., Miller, N. J., G. Bolwell, P. & Rice-Evans, C. A. (1997) Antioxidant activity of phytoestrogenic isoflavones. *Free Radical Research*, *26*(1), 63-70.

Rumsey, G. L., Siwicki, A. K., Anderson, D. P., & Bowser, P. R. (1994). Effect of soybean protein on serological response, non-specific defense mechanisms, growth, and protein utilization in rainbow trout. *Veterinary Immunology and Immunopathology*, *41*(3-4), 323-339.

Saha, N. R., Usami, T., & Suzuki, Y. (2003). A double staining flow cytometric assay for the detection of steroid induced apoptotic leucocytes in common carp (*Cyprinus carpio*). *Developmental & Comparative Immunology, 27*(5), 351-363.

Sarà, G., Mangano, M. C., Johnson, M., & Mazzola, A. (2018). Integrating multiple stressors in aquaculture to build the blue growth in a changing sea. *Hydrobiologia*, *809*(1), 5-17.

Sarker, P. K., Kapuscinski, A. R., Lanois, A. J., Livesey, E. D., Bernhard, K. P., & Coley, M. L. (2016). Towards sustainable aquafeeds: Complete substitution of fish oil with marine microalga *Schizochytrium* sp. improves growth and fatty acid deposition in juvenile Nile tilapia (*Oreochromis niloticus*). *PLoS One*, *11*(6), 1-17

Si, H., & Liu, D. (2007). Phytochemical genistein in the regulation of vascular function: new insights. *Current Medicinal Chemistry, 14*(24), 2581-2589.

Singh, N., & Turner, A. (2009). Leaching of copper and zinc from spent antifouling paint particles. *Environmental Pollution*, *157*(2), 371-376.

Sumaila, U. R., Bellmann, C., & Tipping, A. (2016). Fishing for the future: an overview of challenges and opportunities. *Marine Policy*, *69*, 173-180.

Suresh, A., & Lin, C. K. (1992). Effect of stocking density on water quality and production of red tilapia in a recirculated water system. *Aquacultural Engineering*, *11*(1), 1-22.

Tandon, V., Pal, P., Roy, B., Rao, H. S., & Reddy, K. S. (1997). In vitro anthelmintic activity of root-tuber extract of *Flemingia vestita*, an indigenous plant in Shillong, India. *Parasitology Research, 83*(5), 492-498.

Tang, F., Li, W., Zhou, X., Liu, Y., Li, Z., Tang, Y., Kou, X., Wang, S. D., Bao, M., Qu, L. D., Li, M., & Li, B. (2014). Puerarin protects against *Staphylococcus aureus*-induced injury of human alveolar epithelial A549 cells via downregulating alpha-hemolysin secretion. *Microbial Drug Resistance, 20*(4), 357-63.

Torno, C., Staats, S., de Pascual-Teresa, S., Rimbach, G., & Schulz, C. (2018). Effects of resveratrol and genistein on growth, nutrient utilization and fatty acid composition of rainbow trout. *Animal: An International Journal of Animal Bioscience,* 1-8.

Treoll, M., Naylor, R. L., Metian, M., Beveridge, M., Tyedmers, P. H., Folke, C., Arrow, K. J., Barrett, S., Crepin, A., Ehrlich, P. R., Gren, A., Kautsky, N., Levin, S. A., Nyborg, K., Osterblom, H., Polasky, S., Scheffer, M., Walker, B. H., Xepapadeas, T., & de Zeeuw, A. (2014). Does aquaculture add resilience to the global food system? *Proceedings of the National Academy of Sciences of the United States of America*, *111*(37), 13257-13263.

Turan, F. (2006). Improvement of growth performance in tilapia (*Oreochromis aureus* Linnaeus) by supplementation of red clover (*Trifolium pratense*) in diets. *Israeli Journal of Aquaculture/Bamidgeh, 58*(1), 34-38.

Turan, F., & Akyurt, I. (2005). Effects of red clover extract on growth performance and body composition of african catfish *Clarias gariepinus*. *Fisheries Science, 71*(3), 618-620.

Vanhonacker, F., Altintzoglou, T., Luten, J., & Verbeke, W. (2011). Does fish origin matter to European consumers? *British Food Journal*, *113*(4), 535-549.

Vaughan, D. S., Yoshiyama, R. M., Breck, J. E., & Angelis, D. L. (1982). Review and analysis of existing modeling approaches for assessing population-level effects of multiple stresses on fish and shellfish. Oak Ridge National Laboratory Publication 1979, Oak Ridge, TN: Oak Ridge National Lab, l-36.

Verdrengh, M., Jonsson, I. M., Holmdahl, R., & Tarkowski, A. (2003). Genistein as an anti-inflammatory agent. *Inflammation Research, 52*(8), 341-6.

Wang, H., Ren, L., Yu, X., Hu., J., Chen, Y., He, G., & Jiang, Q. (2017). Antibiotic residues in meat, milk and aquatic products in Shanghai and human exposure assessment. *Food Control*, *80*, 217-225.

Wang, M. & Lu, M. (2016). Tilapia polyculture: a global review. *Aquaculture Research*, *47*, 2363-2374.

Watts, J. E. M., Schreier, H. J., Lanska, L., & Hale, M. S. (2017). The rising tide of antimicrobial resistance in aquaculture: sources, sinks and solutions. *Marine Drugs*, *15*(6), 1-17.

Wendelaar Bonga, S. E. W. (1997). The stress response in fish. *Physiological Reviews*, *73*(3), 591-625

Workagegn, K. B. (2012). Evaluation of growth performance, feed utilization efficiency and survival rate of juvenile nile tilapia, oreochromis niloticus (linnaeus, 1758) reared at different water temperature. *International Journal of Aquaculture, 2*(9)

Wu, L., Qiao, H., Li, Y., & Li, L. (2007). Protective roles of puerarin and Danshensu on acute ischemic myocardial injury in rats. *Phytomedicine: International Journal of Phytotherapy and Phytopharmacology, 14*(10), 652-658.

Wu, Y., Kaiser, H., & Jones, C. L. W. (2019). A first study on the effect of dietary soya levels and crystalline isoflavones on growth, gonad development and gonad histology of farmed abalone, *Haliotis midae*. *Aquaculture International, 27*(1), 167-193.

Xiao, J., Cao, H., Wang, Y., Zhao, J., & Wei, X. (2009). Glycosylation of dietary flavonoids decreases the affinities for plasma protein. *Journal of Agricultural and Food Chemistry, 57*(15), 6642-6648.

Yanagihara, K., Ito, A., Toge, T., & Numoto, M. (1993). Antiproliferative effects of isoflavones on human cancer cell lines established from the gastrointestinal tract. *Cancer Research*, *53*, 5815-5821.

Yue, P., Yuan, H.H., Zhu, W., Cong, L., Xie, H., Liu, Z., Wang, L., & Xiao, X. (2008). The study to reduce the hemolysis side effect of puerarin by a submicron emulsion delivery system. *Biological & Pharmaceutical Bulletin*, *31*(1), 45-50.

Yue, X., Abdallah, A. M., & Xu, Z. (2010). Distribution of isoflavones and antioxidant activities of soybean cotyledon, coat and germ. *Journal of Food Processing and Preservation, 34*(5), 795-806.

Zhou, C., Lin, H., Ge, X., Niu, J., Wang, J., Wang, Y., Chen, L., Huang, Z., Yu, W., & Tan, X. (2015). The effects of dietary soybean isoflavones on growth, innate immune responses, hepatic antioxidant abilities and disease resistance of juvenile golden pompano *Trachinotus ovatus*. *Fish & Shellfish Immunology*, *43*(1), 158-166.

Zhou, C., Lin, H., Huang, Z., Wang, J., Wang, Y., & Yu, W. (2015). Effects of dietary soybean isoflavones on non-specific immune responses and hepatic antioxidant abilities and mRNA expression of two heat shock proteins (HSPs) in juvenile golden pompano *Trachinotus ovatus* under pH stress. *Fish & Shellfish Immunology*, *47*(2), 1043-1053.