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Effects of Dietary Genistein and Fugacity Modelling in an Aquaculture System

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EFFECTS OF DIETARY GENISTEIN AND FUGACITY MODELLING IN AN AQUACULTURE SYSTEM

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DISSERTATION ACCEPTANCE PAGE
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This dissertation is approved as a creditable and independent investigation by a candidate for the Doctor of Philosophy degree and is acceptable for meeting the dissertation requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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> [20]Now to Him who is able to do far more abundantly than all that we ask or think, according to the power at work within us, [21]to Him be glory in the church and in Christ Jesus throughout all generations, forever and ever. Amen. (Ephesians 3:20-21). Reference: Crossway. (2023). The ESV Bible. https://www.esv.org/Ephesians+3/

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ABSTRACT

EFFECTS OF DIETARY GENISTEIN AND FUGACITY MODELLING IN AN AQUACULTURE SYSTEM

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Fish production from aquaculture has continued to grow over the past several decades, with finfishes being fed formulated, fish meal-based diets supporting most of that industry's growth. However, increased demand and costs, coupled with static volume and erratic supplies of fish meal, has resulted in the use of alternative plant-derived protein sources. A common oilseed protein source is soybean meal (SBM), because of its protein content and amino acid profile; however, it also contains multiple anti-nutritional factors, including phytoestrogens. Phytoestrogens are naturally produced, estrogen-mimicking compounds, with isoflavones receiving the most investigation as higher levels of these compounds occur within SBM. Isoflavone content of SBM can vary widely based on numerous factors from varieties and growing conditions to processing effects. A wide array of *in vivo* and *in vitro* studies on isoflavones have been performed on numerous fish species, with results ranging from competition for estrogen receptor sites to intersex characteristics in gonads. Likewise, a variety of different factors (i.e., phytoestrogen compounds, inclusion levels, species, life stage, etc.) and various combinations appeared to influence the potential effects (if any) of phytoestrogens upon finfishes with no distinct relationships occurring among species or with specific phytoestrogens or inclusion levels were discovered in the literature.

To study the effects of the isoflavone genistein on growth, condition, and estrogenic responses of first-feeding Rainbow Trout (*Oncorhynchus mykiss*; initial weight = 109 ± 22 mg [mean ± SD]), a 112-day feeding trial was performed by impartially and randomly stocking larvae into 24, 110-L glass aquaria providing six replicate aquaria per treatment. The larvae were fed one of four experimental diets containing the following SBM:genistein (g/100 g) ratios: 0:0 (Ref), 20:0 (NoGen), 20:0.124 (LoGen), and 20:0.249 (HiGen). Individual mean total length and weight was measured at 2-week intervals for the duration of the feeding trial. Plasma vitellogenin (VTG) was measured from blood samples collected nine days after feeding trial termination. Growth and condition parameters were significantly higher for fish fed the Ref diet, while growth was significantly lower for HiGen when comparing SBM-based diets only. Hepatosomatic index values and mortality rates were not significantly different. Plasma VTG levels formed a U-shaped response curve with fish fed NoGen and HiGen diets having statistically lower and higher plasma levels, respectively, while those fed Ref and LoGen diets were similar. These results indicated that SBM inclusion and increasing levels of dietary genistein did negatively impact growth and condition of first-feeding Rainbow Trout. Also, the U-shaped VTG response curve lends further evidence to the dualistic role of genistein as an estrogen agonist and antagonist.

While numerous studies have looked at the various biological and physiological effects of dietary and water-borne exposure to genistein and other phytoestrogens, not much emphasis has been placed on the incorporation and/or pathways of such chemicals within and out of aquaculture systems. Likewise, the use of fugacity models to depict chemical partitioning within such systems remains limited. As such, genistein

concentrations were measured in the culture water and fish compartments of 18, 110-L microcosms containing larval Rainbow Trout fed the same experimental diets as mentioned above, except for the LoGen treatment that was removed from analyses due to funding constraints. Genistein concentrations were measured in whole-body fish tissue samples, fish livers, fecal material, and culture water samples at mid- (56 or 63 days of experiment [DOE]) and end-points (112 or 119 DOE). Significant differences occurred between the mid- and end-point measurements for all four parameters along with statistically significant differences among all treatments for whole-body, fecal, and livers samples. For water samples, the Ref treatment was statistically lower than both the NoGen and HiGen. Level I and II fugacity models consisting of three environmental compartments (water, fish, and air) were developed using parameters derived from the literature. Estimated compartment concentrations were determined at both the mid- and end-points using genistein amounts from the last 2 weeks of feeding (L2W) or total amounts fed (TGA) to each study point. The model estimates were then compared against the empirically derived data using percent bias (PBIAS) to determine goodness-of-fit of the models resulting in a wide range of PBIAS values. For Level I models, PBIAS ranged from (-2,370 to 96%) with several models falling in the acceptable PBIAS range (-70 to 70%). For Level II models, all models underestimated and were >94% PBIAS except for water at the mid-point using TGA data. Genistein concentrations in the samples indicated that over time, the fish appeared to excrete larger amounts of the genistein, thereby, increasing concentrations within the culture water, most likely due to ontogenetic changes. The concentrations within the culture water were similar to genistein levels seen from other effluent sources and at levels deemed biologically relevant; therefore, dietary

genistein from aquaculture diets could be considered a contaminant of emerging concern. Results from the fugacity models indicated that, while relatively simplistic in nature and capable of determining compartment partitioning, several necessary assumptions needed for Level I and II fugacity models were unmet resulting in poor compartment concentration estimates. However, PBIAS results from several models did show acceptable estimates meriting further investigation into the use of such models due to the relative simplicity and ease of use.

CHAPTER 1. PHYTOESTROGENS AND FUGACITY MODELS IN AQUACULTURE

Over the past seven decades, the production of food fish from aquaculture on a world-wide basis has expanded faster than any other major food production sector, with an average annual growth of 3.3% from 1950 to 2018 (FAO, 2022). In part, this continued growth has occurred due to the static capture fishery production since the late 1980's to accommodate human consumption demands. In 2020, 178 million metric tons of food fish, with an estimated value of $406 billion USD, was produced; farmed food fish accounted for 88 million metric tons ($265 billion USD) of total production (FAO, 2022). From 2000 to 2016, aquaculture yield has risen from 25.7 to 46.8% of combined capture fisheries and aquaculture global production (FAO, 2018). The Food and Agricultural Organization (FAO) indicated that absolute production of total food fish production was achieved without the use of feed (e.g., unfed species) remained relatively stable (FAO, 2022). However, the overall proportion of unfed species total production showed a decreasing trend from over 40% before 2000 to 27.8% from in 2020 (Figure 1-1) (FAO, 2022). This trend reflected the faster growth of the feed-fed species sector, due to the development and improved availability of formulated aquafeeds (FAO, 2018).

Fish meal is considered one of the most complete nutritional and digestible ingredients used in aquafeeds, due to high levels of dietary essential amino acids, fatty acids, digestible energy, vitamins, and minerals (Abdelghany, 2003; FAO, 2018; Lech & Reigh, 2012). However, fish meal production has seen an overall declining trend since 1994, due to fish meal demand and over-fished marine stocks resulting in higher prices (FAO, 2022). In response, there has been an increase in research and use of alternative

protein sources (Coyle et al., 2004; FAO, 2022; Lech & Reigh, 2012). One common, alternative plant-based protein often used in aquafeeds is soybean meal (SBM). Soybean meal is considered one of the most important and highly used plant-based protein sources for partial or entire replacement of fish meal, due to its high protein content, amino acid profile, global availability, and affordability (Hertrampf & Piedad-Pascual, 2000; Lech & Reigh, 2012). Similar to the growth in global aquaculture, global production of SBM has continued to increase from 234 to 251 million metric tons from 2018/2019 to 2022/2023, with current production levels at 247 million metric tons as of April 2022/2023 (USDA, 2023b). The United States alone has seen an average annual increase from 22 to 47 million metric tons in SBM production from 1980 to 2022/2023 (USDA, 2018, 2023a) and is estimated to reach 53.7 million metric tons by 2032/33 (USDA, 2023c).

With any plant-based protein source, there is usually a suite of compounds generally classified as anti-nutritional factors. In particular, SBM contains several anti-nutritional factors, including: protease inhibitors, lectins, antigenic proteins, phenolic compounds, oligosaccharides, and phytates (Liener, 1989). Other potentially negative compounds found in SBM include phytoestrogens, which are natural estrogenic compounds found in plants and are broadly classified by their chemical structures as isoflavones, coumestans, and lignans (Green & Kelly, 2009; Kurzer & Xu, 1997). Classically defined, phytoestrogens describe compounds with estrogenic effects on the central nervous system such as inducing estrus and growth stimulation of the female genital tract in animals (Lieberman, 1996); however, a broader definition also includes chemicals with suggestive estrogenic characteristics, such as binding to the estrogen receptor and/or conversion into compounds with estrogenic effects (Francis et al., 2001;

Kurzer & Xu, 1997). Soybeans, and their associated SBM products, contain various isoflavones and derivatives which have been shown to be bioavailable, detectable in blood plasma, and produce estrogenic effects in fish (Bennetau-Pelissero et al., 2001; Green & Kelly, 2008, 2009; Pelissero et al., 1991). The unconjugated forms (i.e., aglycones) are genistein, daidzein, and glycitein with each of these existing in other chemical forms of glucoside conjugates (Kurzer & Xu, 1997; Villares et al., 2011). Other derivatives include formononetin and biochanin A (Francis et al., 2001; Kurzer & Xu, 1997). Along with these isoflavones, coumestan occurs in two forms and two plant lignans, capable of conversion to mammalian lignans resulting from bacterial action in the gastrointestinal tract (Kurzer & Xu, 1997). Similarly, it has been shown that when enzymatic metabolism occurs in the digestive tract of laboratory rats, mimicking human gut microflora, isoflavone glucosides release their respective aglycone derivatives (Bowey et al., 2003); however, no apparent evidence of this conversion or enzymatic action has been demonstrated for finfishes. Likewise, another study has shown that aglycones are primarily absorbed within the human digestive tract (Izumi et al., 2000). Therefore, physiological effects in fishes appear to be primarily associated with aglycones (Peñalvo et al., 2004).

Due to the increased production forecast for aquaculture finfishes and potential use of SBM, it becomes apparent that large quantities of SBM or refined SBM products will likely be used in formulated aquaculture feeds. As a result, phytoestrogens found in SBM will be introduced into aquaculture systems and cultured finfish populations. While several studies have been undertaken to determine the effects of phytoestrogens on various cultured finfishes, including Rainbow Trout (*Oncorhynchus mykiss*) (Bennetau-

Pelissero et al., 2001; Kaushik et al., 1995; Mambrini et al., 1999), Channel Catfish (*Ictalurus punctatus*) (Green & Kelly, 2009) and Goldfish (*Carassius auratus*) (Bagheri et al., 2014; Ishibashi et al., 2002), there remains a large void of the potential effects that individual and combined phytoestrogens may have on different cultured species and life stages. Similarly, no studies have looked at the potential discharge of isoflavones into natural ecosystems from SBM-based feeds used in aquaculture settings. However, several studies have looked at the introduction and fate of various phytoestrogens in aquatic environments including river waters (Kawanishi et al., 2004), wastewater effluents (Liu et al., 2010), and sediments (Kelly et al., 2015) along with potential effects on aquatic organisms (Kelly et al., 2014; Rearick et al., 2014; Kelly et al., 2015). It would be highly unlikely that all phytoestrogens present in SBM aquaculture diets would be incorporated into the cultured fish tissue; therefore, it becomes important to determine the fate of these un-incorporated isoflavones (e.g., fecal matter, feed leaching, and waste feed) within aquaculture settings and their potential pathways to natural ecosystems.

One tool for determining the potential concentrations and fate of chemicals of interest would be environmental models and more specifically, fugacity models. Fugacity models are widely used in chemical process calculations by utilizing thermodynamic equilibria criteria and partitioning calculations, which mathematically describe the rates of chemical transportation and/or diffusion between phases such as: air-water, water-biota, soil-air, etc. (Mackay 2001). The fugacity concept provides a practical modeling approach to determine rate transfers between source and destination phases, and transforms complex chemical equations (i.e., chemical reactions, advective flows, non-diffusive transport rates, etc.) and chemical behaviors in multiphase environments into

more easily determined and calculated fugacity expressions and equations (Mackay 2001). Fugacity models have been developed and used for a wide variety of natural, laboratory, and hypothetical multimedia environments ranging in scales from bioaccumulation within a single organism to chemical fates within lakes and rivers, to global models (Mackay 2001). However, no apparent model has been developed, or at least reported in the literature, specifically for aquaculture systems. This is surprising as such systems provide ideal parameters and an environment for such models which are not normally available when considering natural environments or systems. For instance, most environmental models must estimate the volume of the incorporated compartments such as fish biomass, flow rates, suspended sediments, etc., while most aquaculture systems, especially intensive tank culture systems, provide all the necessary data through normal aquaculture practices (i.e., weighing fish/tanks, water quality analysis, tanks size, system flow rates, feed composition, and feeding rates, etc.). While some previous fugacity models have been developed and possibly could be adapted to work with aquaculture systems, one of the primary areas of interest that has yet to be studied is the interactions of chemicals within aquaculture diets and examined in various phases (i.e., fish and water) within the aquaculture system. These interactions become of primary interest due to the possibility of determining bioaccumulation (i.e., net consequence of uptake, biotransformation, and elimination of a chemical by organisms [Mackay, 2001; Newman & Unger, 2003]), with corresponding potential effects upon cultured fish, and possible chemical concentrations that may be transferred from aquaculture to natural systems via discharge water.

Therefore, the overall goal of this dissertation research was to determine the levels and interactions of the aglycones, with genistein holding primary interest, from their initial concentrations in a SBM product to fates within an aquaculture system. To accomplish this goal, the following objectives of this proposed research were to determine 1) the concentrations of several phytoestrogens found in several commercial SBM products and subsequent aquaculture diet compositions, 2) potential performance, physiological, and/or morphological effects on rainbow trout fed various levels of genistein, and 3) determine the potential fate and estimate concentrations of genistein in an aquaculture system using a fugacity-based model, which are then compared against concentrations determined empirically.

Null Hypotheses and Research Objectives:

H_{O1}: There are no differences in phytoestrogen concentrations from different SBM products manufactured with different methods.

Objective 1: To determine the concentrations of phytoestrogens found in a variety of different SBM feed products used in aquaculture feeds.

Strategy 1: Phytoestrogen concentrations will be analytically determined in different SBM products manufactured from different methods.

H_{O2}: There are no differences in growth performance, physiology, and/or morphology of cultured Rainbow Trout fed experimental diets containing increasing levels of genistein and a reference diet (i.e., no SBM and genistein present).

Objective 2: To determine the potential performance, physiological, and/or morphological effects on Rainbow Trout fed experimental diets containing increasing levels of genistein and a reference diet.

Strategy 2: A feeding trial will be performed where Rainbow Trout will be fed experimental diets containing increasing levels of genistein and a reference diet and performance, physiological, and/or morphological effects will be measured and statistically analyzed for differences.

H_{O3}: There are no differences between empirical genistein concentrations and estimated concentrations from fugacity models for fish and water compartments within an aquaculture system.

Objective 3: To determine the accuracy of proposed fugacity models specifically developed to determine chemical partitioning and estimated concentrations within a flow-through aquaculture system.

Strategy 3: Level I and II fugacity models will be developed utilizing available information found within the literature and experimentally derived to estimate the amount of genistein found within various compartments of an aquaculture system. A feeding trial (mentioned in Strategy 2 above) will be performed to provide empirical genistein data for comparison to model estimates to determine model goodness-of-fit.

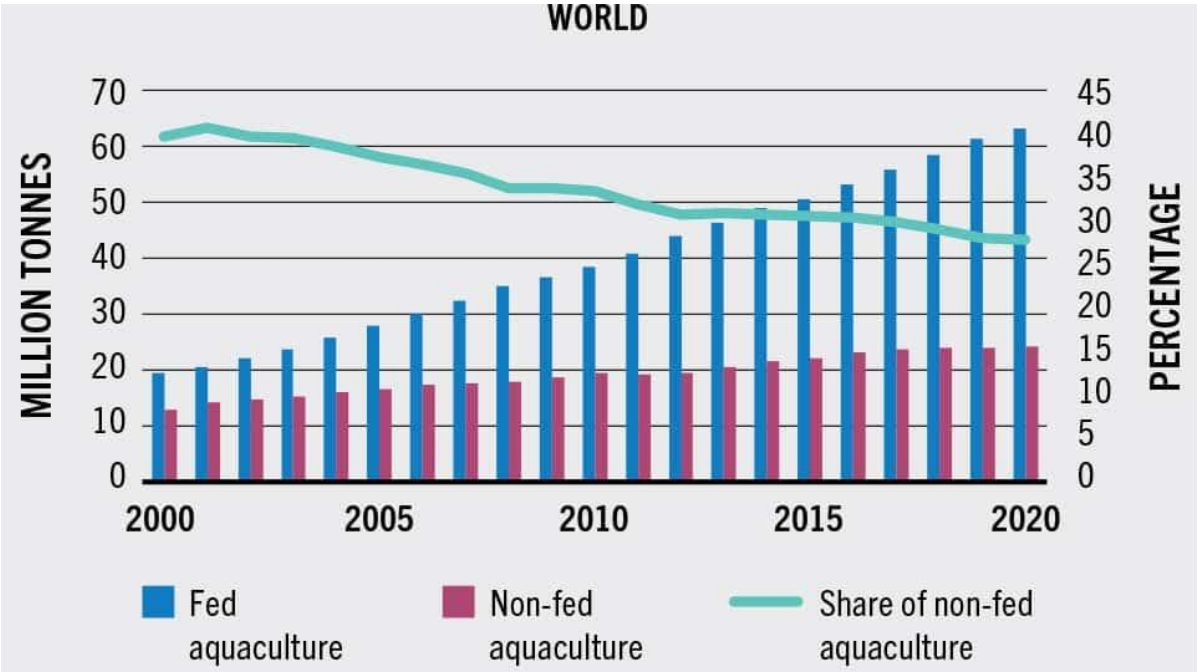

Figure 1-1. Total world production of fed and unfed aquatic animal species and the trend line for the percent share of production unfed species contribute to the total world production. Figure from FAO (2022).

CHAPTER 2. SOYBEAN MEAL PROCESSING AND ITS EFFECTS ON SOYBEAN MEAL PHYTOESTROGEN TYPES, LEVELS, AND THEIR ESTROGENIC EFFECTS ON FINFISHES

Soybean Meal Processing

Soybeans have a two-fold value as an oil crop and a protein crop with soy protein products accounting for roughly two-thirds of the overall value (Johnson & Smith, 2013). Currently, there are three processing methods used for oil extraction and soybean meal production. One of the first commercial processes developed and still used to a limited degree today is mechanical extraction of oil from soybeans with hydraulic or screw presses (Johnson & Smith, 2013). The screw-press process involves heating or cooking whole soybeans, then mechanically extracting the oil (Johnson & Smith, 2013). Several anti-nutritional factors are reduced or destroyed by the friction heat of the screw presses but this type of SBM has higher levels of residual oil, lower protein contents, greater rumen by-pass values, and higher palatability compared to other oilseed meals, such as copra, cottonseed, peanut, rapeseed, and sunflower seed (Johnson & Smith, 2013; USDA, 2023b). These factors allow for the meal to be used in dairy rations to help balance amino acid profiles of rations including alfalfa forage and corn-based byproducts (Johnson & Smith, 2013). Similar to the screw-press process, extruding-expelling has been developed where prior to oil extraction by screw presses, a dry extruder is used to process raw soybeans eliminating the need for steam generation for heating or cooking (Johnson & Smith, 2013). These plants are generally farmer-owned and relatively small, typically processing 4.5 to 22.7 metric tons of soybeans per day, and ideally suited to provide the following: meal to nearby livestock feeders, identity preserved processing, marketing

certified organic or non-genetically modified products, and/or SBM and oils with specifically enhanced traits (Johnson & Smith, 2013).

The third and most widely used process used is solvent extraction with more than 99% of the United States soybeans being processed with this method as several soybean plants have shown processing rates of more than 2,700 metric tons of raw soybeans per day (Johnson & Smith, 2013). Due to the high use of this method and its ability to produce high quality SBM consistently, this method and the resulting SBM derivatives will be the focus of this dissertation research as it currently provides the base for primary SBM ingredients used in aquaculture diets. As compared to screw-press and extruding-expelling processes, the solvent extraction process consists of several more handling steps due to the use of chemicals for oil extraction. First, raw soybeans are cleaned to remove any foreign material, dried to approximately 9.5% moisture to loosen the hull and improve de-hulling, and then passed through the corrugated roller mills to crack soybeans into 8 to 16 pieces (Johnson & Smith, 2013). Second, the soybeans are cleaned a second time using aspiration with high flow rates, which separates materials according to terminal velocity, a function of kernel density, shape, and surface roughness (Hurburgh et al., 1996). The soybean hulls separated and removed by aspiration provide de-hulled soybeans (‘meats’) which are then passed over a gravity table to allow small particles that were aspirated along with hull fractions to be salvaged (Johnson & Smith, 2013). By removing the hulls and fractionating, greater oil extraction can be obtained due to less total volume to process, and increases the protein content of the SBM (Johnson & Smith, 2013). Third, cracked soybean meats are then heated to approximately 74°C to condition the meats prior to flaking; meats not properly cracked and conditioned may not achieve

desired cell rupture or distortion necessary for efficient oil extraction and/or produce an excess of fine material that interferes with extraction (Johnson & Smith, 2013). Cracked and heated meats are then passed through smooth surfaced rolls in a roller mill to produce flakes roughly 0.25 to 0.30 mm thick in a process called flaking (Johnson & Smith, 2013). Fourth, flakes are then transferred either directly to the extractors or passed through an expander for additional treatment (Johnson & Smith, 2013). Expanders are similar to extruders and produce porous pellets from flakes with increased cell rupturing and greater density allowing for increased ease of oil extraction, drainage of solvents thereby reducing evaporating efforts, and through-put capacity of the extractor (Johnson & Smith, 2013). Several types of extractors exist but all operate similarly: 1) solvent is percolated through a bed of flakes and/or expanded material, 2) flakes are washed with hexane in a counter-current fashion which causes the soybean lipid to solubilize, 3) the hexane-oil mixture is separated through a series of steps to produce an oil-rich extract called miscella, and 4) the miscella is evaporated and the solvent recycled to the extractor (Johnson & Smith, 2013). Fifth, spent flakes (i.e., soybean flakes with the oil removed) are transferred to desolventizer-toaster to remove any excess hexane by heating the spent flakes to evaporate the hexane and sparged with steam to vaporize residual hexane (Johnson & Smith, 2013). This process, called toasting, also reduces activity of urease and trypsin inhibitors, which may reduce digestibility and nutritional value of the SBM product (Johnson & Smith, 2013). Sixth, the resulting meal is transferred to a dryer-cooler to reduce the moisture content to approximately 13 to 14% and cooled for storage. Finally, the meal is screened and ground to uniform particle size with a hammer mill before shipping (Johnson & Smith, 2013). The finished SBM from this process is referred

to as de-hulled SBM or high protein meal soybean hulls or soybean mill-run can be added back to this product to adjust protein levels and meet end-user specifications (Johnson & Smith, 2013).

Soybean Phytoestrogens

Types and Content of Phytoestrogens in Soybean Products

Phytoestrogens include over 100 molecules that can be classified into four groups according to their chemical structure: isoflavones, coumestans, lignans, and stilbens with isoflavones and coumestans identified as the most common estrogenic compounds in plants (Kurzer & Xu, 1997; Pilšáková et al., 2010). Isoflavones are found almost exclusively in legumes (Setchell & Cassidy, 1999), while numerous coumestans have been isolated from other plant species; however, only a limited number of these have been shown to possess uterotropic activity (Kurzer & Xu, 1997). Lignans are considered phytoestrogens by some researchers due to their estrogen-like actions, but they have not been shown to induce estrus (Kurzer & Xu, 1997). Stilbens, specifically resveratrol, can function as natural antioxidants (Pilšáková et al., 2010).

Soybeans are one of the most abundant and significant dietary sources of isoflavones and contain various isoflavone derivatives (Kurzer & Xu, 1997; Pilšáková et al., 2010; Setchell & Cassidy, 1999). The unconjugated forms (i.e., aglycones) are genistein, daidzein, and glycitein with each of these also being found as a glucoside form (i.e., genistin, daidzin, and glycitin), acetylglucoside, and malonyglucoside (Kurzer & Xu, 1997; Villares et al., 2011). Other derivatives include formononetin and biochanin A (Francis et al., 2001; Kurzer & Xu, 1997). Along with isoflavones, coumestans and lignans are also found in soybeans. Coumestan occurs in two forms, coumestrol and 4’-

methoxycoumestrol, in soybeans (Kurzer & Xu, 1997; Setchell & Cassidy, 1999). Two plant lignans, secoisolariciresinol and matairesinol, are converted to the lignans, enterodiol and enterolactone, respectively, in mammals from digestion in the gastrointestinal tract. Secoisolariciresinol is transformed via hydrolysis of the sugar moiety, dehydroxylation, and demethylation, while matairesinol via dehydroxlation and demethylation (Kurzer & Xu, 1997; Setchell & Cassidy, 1999). Similarly, within the gastrointestinal system, bacterial enzymes catalyze isoflavones into various derivatives via reduction, deoxygenation, hydroxylation, and C-ring cleavage (genistein only) (Setchell, 2017). Daidzein also undergoes metabolism and biotransformation by more than 10 different intestinal microbes to produce equol, including but not limited to, *Adlercreutzia equolifaciens, Asaccharobacter celatus, Bacteroides ovatus, Finegoldia magna*, *Lactobacillus mucosae*, *Slackia equolifaciens, S. isoflavoniconvertens*, and *Streptococcus intermedius*; however, a significantly higher abundance and prevalence of *A. celatus* and *S. isoflavoniconvertens* occurred in humans labelled “equol producers” (Iino et al., 2019). Equol has a similar chemical structure to estradiol (E2) and an estrogenic potency an order of magnitude higher than daidzein (Figure 2-1) (Kurzer & Xu, 1997; Setchell & Cassidy, 1999). Oilseeds, including soybeans, provide the richest plant sources of lignans, but its concentration in foods has not been fully characterized (Kurzer & Xu, 1997). Both isoflavones and lignans show similar patterns of metabolism by bacteria in the gastrointestinal tract and disposition in mammals (i.e., humans and rats) (Kurzer & Xu, 1997).

Isoflavone content in soybeans and soybean products can vary based on numerous factors such as soybean varieties, identical varieties grown at different environments,

different growing seasons at the same location, (Eldridge & Kwolek, 1983; Wang & Murphy, 1994) and processing procedures used. One study analyzed six SBM isoflavones (daidzin, glycitin, genistin, daidzein, glycitein, and genistein) derived from the following scenarios that included: 1) four different varieties, 2) two varieties grown in four different locations, and 3) one variety over a four-year period. Total isoflavone content among the four varieties ranged from 115.9-309.3 mg/100 g with genistein and daidzein ranges of 2.5-4.6 and 0.8-3.2 mg/100 g, respectively (Eldridge & Kwolek, 1983). Two varieties raised at four different sites had 46.9-195.1 mg/100 g of total isoflavone content with genistein and daidzein ranges of 0.03-1.0 and 0.5-3.5 mg/100 g, respectively (Eldridge & Kwolek, 1983). Across a four-year period, isoflavone content ranged from 245.2-362.5 mg/100 g with genistein and daidzein ranges of 0.2-0.9 and 0.8-2.3 mg/100 g, respectively, and further showed that soybean hulls contributed small amounts of isoflavones (<10%) across varieties (Eldridge & Kwolek, 1983). This study also compared initial processing steps effects upon isoflavone content of two tested soybean varieties by comparing cracked, dehulled, flaked, and pentane-hexane extracted soybeans against full-fat (i.e., unextracted) soybean flakes (Eldridge & Kwolek, 1983). Results indicated that de-hulling cracked soybeans and defatting full-fat soybean flakes did not remove or greatly affect the isoflavone content from the subsequent soybean meal (Eldridge & Kwolek, 1983).

Processing Effects on Isoflavone Content

Similar to factors mentioned above, isoflavone content and composition can be significantly affected through loss or changed into different derivatives based on the processing procedures used (Setchell, 2017). Fermentation is one of the primary

processing steps which causes such significant changes; whereby, soybean products used for human consumption (e.g., tempeh) contain greater levels of aglycones compared to other forms of isoflavones because of the enzymatic hydrolysis and transportation with the protein fraction into the resulting soybean product (Kurzer & Xu, 1997; Setchell, 2017; Setchell & Cassidy, 1999; Wang & Murphy, 1996). In general, whole soy foods contain higher levels (3.0-3.5 mg isoflavones/g) versus those made from purified soy proteins (1.0-1.5 mg/g) (Setchell, 2017). Studies have be conducted to examine isoflavone content and composition of various fermented and non-fermented soy foods used for human consumption along with processing effects (Chen & Wei, 2008; Wang et al., 1998; Wang & Murphy, 1996; Wang & Murphy, 1994). However, as most of these soybean products are not used in aquaculture, such information will not be presented here and the readers are encouraged to see the above-mentioned citations for more details on this topic. However, useful information, such as study methods, from selected studies is relevant to provide an understanding and framework for future research on isoflavones within aquaculture diets. Hence, two studies are reviewed below to provide insight into methods for such research.

A study by Wang and Murphy (1996) utilized a mass balance approach to determine processing effects on the isoflavone content of tempeh, soymilk, tofu, and soy protein isolate. In their preliminary study, Wang and Murphy (1996) produced each soybean product using laboratory or pilot plant methods and sampled products at each processing step. Samples analyzed included: raw, de-hulled, soaked, and cooked soybeans; soaking and cooking water; seed coats; and final products (Wang & Murphy, 1996). All samples were freeze-dried to determine moisture content for determination of

isoflavone concentrations on a dry matter basis following isoflavone extraction methods described in Wang and Murphy (1996) and high-pressure liquid chromatography (HPLC) analysis described in Wang and Murphy (1994). The different production processes, primarily fermentation, chemical coagulation, and pH precipitation, resulted in differing isoflavone loss rates and redistribution. Results indicated that for tempeh, each processing step resulted in isoflavone losses with a cumulative loss of 76%, primarily from soaking/de-hulling (12% loss) and cooking steps (49% loss) (Wang & Murphy, 1996). Fermentation did not greatly affect total isoflavone content but did result in isoflavone transformation with an increase in aglycones (i.e., daidzein and genistein) and a corresponding decrease in glycones (i.e., daidzin and genistin) (Wang & Murphy, 1996). For soymilk, no significant isoflavone losses occurred while tofu maintained 33% of its original isoflavone content with the coagulation step attributed as the main source for the loss (Wang & Murphy, 1996). For soy protein isolate, the processing procedure resulted in a significant 53% loss of total isoflavones primarily from the alkaline extraction step where isoflavones transfer into the alkaline-soluble fraction which is usually discarded (Wang & Murphy, 1996). Similarly, the alkaline extraction step increased daidzein, genistein, and glycitein in the soluble portion resulting in higher amounts of these aglycones in the soy protein isolate compared to the soy flour (Wang & Murphy, 1996).

These results led the authors to several conclusions. First, heat treatment/cooking during tempeh production appeared to increase isoflavone leaching rates from the de-hulled soybeans into the cook water but did not further affect isoflavone retention during tofu production or change isoflavone composition distribution (Wang & Murphy, 1996).

Likewise, soaking and cooking during soymilk and tofu production resulted in higher glucoside and aglycone isoflavones; however, the coagulation step for these products had the largest impact on isoflavone losses (Wang & Murphy, 1996). Second, fermentation during tempeh production did not result in significant isoflavone losses but affected redistribution by greatly increasing aglycones over glucosides (Wang & Murphy, 1996) most likely due to the hydrolytic action of β-glucosidase (Murakami et al., 1984). Third, in contrast to Eldridge and Kwolek (1983), no isoflavone extraction occurred when defatting ground soybeans with hexane during soy protein isolate production; instead, major isoflavone loss (≈50%) occurred when defatted soybean flour was extracted with an alkaline solution (Wang & Murphy, 1996). It was hypothesized that an alkaline pH might disrupt isoflavone-protein bonds by changing the charge of one or both components (Wang & Murphy, 1996).

In a later study, Wang et al. (1998) utilized mass balance studies to investigate the effects of processing on isoflavones in the production of soy protein isolate, which constitutes one of the major soy protein products used in the food industry. Defatted soy flour was processed into soy protein isolate via extraction, precipitation, washing, and drying with process samples collected for soy flour and soy protein isolate along with solid waste, whey, and wash water (Wang et al., 1998). Samples were freeze-dried, processed for HPLC analysis, and analyzed for isoflavone content and mass balance calculations (Wang et al., 1998). Soy protein isolate and soy flour contained 1,352 and 1,512 μg/g total isoflavones, 246.08 and 24.17 μg/g genistein, and 125.39 and 0.0 μg/g daidzein, respectively. Total isoflavone contents of solid waste, whey, and wash water were 958, 1,865, and 28,684 μg/g, respectively (Wang et al., 1998). Soy protein isolate

contained higher levels of the aglycones most likely due to hydrolysis of genistin and daidzin found in the soy flour, while the wash water had significantly higher concentrations of all individual and total isoflavones (Wang et al., 1998). Mass balance calculations indicated processing of soy flour into soy protein isolate resulted in 74% loss of isoflavones with solid waste, wash water, and whey accounting for 19.0, 21.6, 14.3%, respectively of the total isoflavones (Wang et al., 1998).

In order to determine differences in phytoestrogen levels between different SBM products, seven different SBMs were obtained and analyzed for several types of phytoestrogens. These SBMs consisted of products that were obtained from either different soybean sources (e.g., genetically modified [GMO] or non-GMO), SBM collected following different processing steps during "normal" SBM production (described above), or specialty SBMs created specifically by companies for use in aquaculture and/or livestock formulated diets. To accomplish this goal, SBMs were collected from different sources, companies, and after different processing steps and then analyzed for a suite of phytoestrogens. Comparisons of the results were made to determine the potential effects processing steps may have on phytoestrogen levels within SBMs, albeit in a cursory manner due to the limited processing information available and limited number of samples analyzed (i.e., no statistical analysis performed).

Methods, Results, and Discussion

Seven different SBMs were obtained from different processing facilities. Solvent extracted SBM, soy white flake, and extruded non-GMO SBM were obtained from the South Dakota Soybean Processors, Volga, South Dakota, processing plant. These SBMs were processed from different soybean sources (i.e., GMO for solvent extracted and soy

white flake vs. extruded non-GMO) and were sampled after different processing steps occurred (Figure 2-2). A detailed description of the soybean processing plant's production steps to create these SBMs was unavailable so all following discussion related to the production and differences of these SBMs will in relation to normal SBM production processes as mentioned previously in this chapter and depicted in Figure 2-2. Similarly, four specialty SBMs produced and marketed specifically for aquaculture and/or livestock diets were obtained from different companies. These SBM products consisted of Soycomil-P® from ADM Animal Nutrition, Quincy, Illinois, ME-PRO® from Prairie Aquatech, Brookings, South Dakota, and HP 300 and HP 330 (not currently produced) from Hamlet Protein Incorporated, Findlay, Ohio. Due to unique processing technologies/techniques and associated trade secrets associated with these products, specific information on processing steps was unavailable with general information for each SBM available on the associated company's website.

All samples were analyzed for phytoestrogen levels of genistein, daidzein, equol, formononetin, and biochanin A by the United States Geological Survey (USGS), Organic Geochemistry Research Laboratory (OGRL), Lawrence, Kansas. The method used for this determination is described in Appendix A. Results from the phytoestrogen analysis are listed in Table 2-1. No statistical analyses were performed as only single samples were analyzed; all comparisons of the different SBMs are observational based on the analyzed data provided from OGRL. However, even without statistical testing, it is apparent that the HP 300 product contained much higher levels of genistein and daidzein with concentrations 21 and 44 times greater, respectively, than the next closest SBM concentration in any other product (Table 2-1).

Based on the phystoestrogen analyses of the different conventional SBMs (i.e., solvent extracted, soy white flake, and extruded), it appeared that soybean source and different processing steps did not result greatly affect phytoestrogen concentrations. For example, the additional steps of desolventization and/or toasting/drying did not greatly affect phytoestrogen levels of solvent extracted and soy white flake compared to extruded non-GMO, which did not undergo such additional processing. This lack of difference most likely occurred for several reasons. First, phytoestrogen levels within the stock soybeans used for SBM production, whether GMO or non-GMO, did not differ greatly. While no samples were collected and analyzed of the raw soybeans used, it is likely that similar levels of phystoestrogens in the raw soybeans occurred and were maintained throughout the initial processing steps of SBM production. Second, the additional steps of desolventizing and toasting/drying did not occur at temperatures high enough to result in phytoestrogen thermal degradation. A study on the thermal stability of genistein and daidzein indicated that both isoflavones were degraded following a thermal treatment of 120°C for 20 min with reductions in residual concentrations of 15-60% from original levels (Ungar et al., 2003). When considering the additional processing steps used for solvent extracted and soy white flake, temperature of the meal products exiting these steps has been reported to be 90-110°C (Kemper, 2021). Therefore, it is plausible that the additional processing steps did not reach temperatures that were high enough and/or for a period long enough to result in noticeable thermal degradation of the measured phytoestrogens within these conventional SBMs as compared to extruded SBM.

When comparing phytoestrogen levels within the specialty SBMs, a large variation in genistein and daidzein concentrations occurred with a range of 0.276-230 and

0.208-591 µg/g, respectively (Table 2-1). As mentioned above, no information on direct processing procedures was obtained for any of these SBMs as they were developed by each company as a distinct, marketable product. However, all of these SBMs were made with intent for use within animal diets/rations (i.e., livestock, poultry, aquaculture, etc.). As such, each company sought to create a product low in ANFs that would be readily used within diets/rations to increase overall digestibility and/or maintain high protein levels within their product as denoted on product descriptions found on each company's website. Therefore, it is apparent that the unique production process of each SBM, even those produced within the same company (i.e., HP 300 vs. HP 330), resulted in differing phytoestrogen levels. As mentioned above in Wang and Murphy (1996), different production processes resulted in differing isoflavone loss rates and redistribution via biotransformation. Therefore, it is most likely that the differing steps and processes used for the HP 300 product resulted in increased biotransformation favoring such isoflavones as genistein and daidzein, loss or biotransformation of these isoflavones during processing, and or conservation of these isoflavones during processing. For both conventional and specialty SBMs, it is apparent that the processing steps used to produce each product affected the overall type, amount, and distribution of phytoestrogens. Future research to determine such effects is warranted. Such information on soybean source (i.e., variety, growth history, etc.) and phytoestrogen concentrations within stock soybeans prior to processing would be beneficial as such information coupled with a mass balance study on the processing steps used in SBM production would help elucidate potential biotransformation and/conservation of phytoestrogens during processing.

Biological and Physiological Effects of Isoflavones on Fishes

General Estrogenic Activity of Isoflavones

As the primary estrogen produced by female fish, 17β-E2 released from the ovaries results in the synthesis of large amounts of the lipoprotein vitellogenin (VTG) within the liver by hepatocytes (Pait & Nelson, 2002). While VTG is primarily related to female fish as a precursor of egg yolk, male fish also possess hepatocyte estrogen receptors (ER) that can synthesize VTG when exposed to E2 or estrogen mimicking compounds (Pait & Nelson, 2002). As such, VTG can be used as a biomarker to determine potential effects of estrogen mimicking compounds upon fish, particularly males, in both field and laboratory studies (Pait & Nelson, 2002). Because of their similar chemical structure with 17β-E2 (Figure 2-1), phytoestrogens can bind with both alpha (ERα) and beta ER (ERβ) (Barnes et al., 2000), which are found in the steroid/thyroid superfamily of intracellular receptors and are primarily located in the nuclear membrane of a variety of cells (Pilšáková et al., 2010). Both types of estrogen receptors (ER) are found within an organism but each has a different role or expression. ERα mediates estrogen actions in the uterus, hypothalamus/pituitary, skeletal, and other estrogen target tissues, while ERβ has roles in the ovary, cardiovascular system, brain, and inflammation response as evidenced in several animal models (Harris, 2007). Interaction between isoflavones and ER results in the activation of estrogen responses within the nuclear membrane, such as transcription processes (Pilšáková et al., 2010). Similar responses to endogenous 17β-E2 may be seen when isoflavone levels reach sufficient levels (approximately > 100 nmol/l for genistein) (Kuiper et al., 1998), but the effects of isoflavones are dependent on the level of endogenous E2, due to competition for estrogen

binding sites (Pilšáková et al., 2010). Genistein and coumestrol have shown affinities to ERβ sites similar to or greater than 17β-E2, with affinities being approximately 3 to 22 times higher for ERβ than ERα, while other isoflavones have affinities ≤200 times lower than 17β-E2 (Kuiper et al., 1998). Likewise, genistein and equol gave a positive response when analyzed with a yeast estrogenicity assay and produced *in vitro* potencies of 2×10^{-3} and 3×10^{-3}, respectively, relative to E2 (Kiparissis et al., 2003). Isoflavones also appeared to have actions like partial estrogen agonists and antagonists, where isoflavones may antagonize and inhibit estrogen action instead of mimicking; this is dependent on numerous factors, such as isoflavone exposure levels, ER numbers, and competition with endogenous E2, etc. (Setchell & Cassidy, 1999). As such, several studies have been performed on a variety of fish species to gain a better understanding of the physiological and growth effects of phytoestrogens. These studies are described in more detail below and are summarized in Appendix B.

Salmonids

Pelissero et al. (1993) performed some of the earliest work to determine the estrogenic potency of phytoestrogens in fish. The authors utilized an *in vitro* technique, wherein, incubated Rainbow Trout (mean weight = 500 g) hepatocytes were exposed to the phytoestrogens genistein, daidzein, coumestrol, equol, formononetin, and biochanin A, along with several estrogen steroids (Pelissero et al., 1993). After exposure, VTG concentrations were analyzed from the various steroids and phytoestrogens and then compared against the potency of E2 (Pelissero et al., 1993). Results indicated that all six phytoestrogens exhibited estrogenic activity but were 1,000-2,000 times less active than E2 (Pelissero et al., 1993). The authors indicated that the phytoestrogens demonstrated

similar estrogenic activity to that of androgens and progestogens; however, they cautioned that the results might not adequately predict the *in vivo* response of these compounds (Pelissero et al., 1993).

Kaushik et al. (1995) performed one of the first studies to investigate potential estrogenic effects of fish fed diets containing SBM. The authors analyzed five different SBM products for phytoestrogens, which are mentioned above in the section "Soybean Phytoestrogens - Types and Content of Phytoestrogens in Soybean Products." The authors performed a 12-week feeding trial with juvenile Rainbow Trout (initial weight = 83 ± 1 g) fed five diets containing either the soy flour (220, 420, and 620 g/kg diet) or SPC (240 and 420 g/kg diet) (Kaushik et al., 1995). Isoflavone levels ranged from 2.9-105.0 mg genistein/kg and 5.4-61.0 mg daidzein/kg in diets. At the end of the growth trial, isoflavone levels of daidzein and genistein in the experimental diets and pooled bile samples from Rainbow Trout were determined, along with plasma VTG concentrations (Kaushik et al., 1995). Isoflavone levels ranged from 2.1-22.6 mg genistein/kg and 2.5-10.6 mg daidzein/kg in trout bile (Kaushik et al., 1995). Rainbow Trout showed no significant differences of VTG levels when fed any of the diets; however, the authors did indicate that higher levels of circulating VTG levels occurred at the end of the feeding trial compared to initial levels (Kaushik et al., 1995). The authors suggested that genistein and daidzein are absorbed by Rainbow Trout and subject to the action of uridine diphosphate glucuronyl transferase and that VTG levels did not appear to be significantly modified from a cultured trout population raised using standard, fish meal based diets and methods (Kaushik et al., 1995).

In another study on Rainbow Trout, Mambrini et al. (1999) sought to determine the effects of feeding differing levels of SPC with DL-methionine supplementation to replace fish meal in high-lipid diets and characterized the SPC source based on anti-tryptic factors and isoflavone (genistein and daidzein) levels. Six experimental diets containing 319.5-635 g SPC/kg diet were fed three times daily for 90 days to trout (initial weight = 106.0 ± 1.9 g SE) held in triplicate tanks (Mambrini et al., 1999). Total isoflavones were extracted from the diets and trout bile, analyzed using HPLC methods, and identified as genistein and daidzein (Mambrini et al., 1999). The base SPC contained high concentrations of genistein (5,903 mg/kg) and daidzein (1,990 mg/kg), while an inverse relationship occurred in bile concentrations with higher daidzein levels (0.51-1.03 mg/kg) compared to genistein (0.21-0.56 mg/kg) (Mambrini et al., 1999). Results indicated that growth rate was strongly reduced by replacing greater than 50% of fish meal with SPC, primarily due to lower feed intake of the SPC-based diets (Mambrini et al., 1999). The authors proposed that isoflavones may have played a role in the lower growth performance by reduced feed intake and/or through negative effects on metabolism. The data suggested that at least part of the isoflavone content was absorbed and metabolized in the digestive tract (Mambrini et al., 1999). It should be noted that a lower daidzein:genistein ratio was measured in the bile compared to the diet, which suggests that genistein was either more available to trout or had better enterohepatic recirculation compared to daidzein (Mambrini et al., 1999). Also, the reduced isoflavone levels within the Rainbow Trout bile could reflect a lack of availability from variation caused by ingredient processing or a faster turnover rate due to the use of a longer feed deprivation period (2 days) (Mambrini et al., 1999)

Bennetau-Pelissero et al. (2001) determined the effects of dietary genistein on endocrine physiology and reproductive functions in Rainbow Trout. Three experimental diets containing 0, 500, and 1,000 mg genistein/kg feed were fed to 6 month-old Rainbow Trout (initial weight = 40 g) for approximately 1 year until spawning occurred (Bennetau-Pelissero et al., 2001). Initial sampling occurred 3 days prior to the start of the feeding trial, followed by every 10 weeks (total of 6 sampling periods) for a wide range of parameters, including: growth (weight), cholesterol, plasma VTG, plasma steroids (testosterone [T], E2, 11-ketotestosterone [11-KT], and 17α,20β$(OH)_2$-progesterone), gonadotropins (beta follicle stimulating hormone [βFSH] and beta lutenizing hormone [βLH]), histological gonad, and spawning indices (sperm volume, milt concentration, sperm motility, egg diameter, weight of ovulated egg, weight of 100 eggs, weight of residual gonad, viability of fertilized eggs, survival at embryo stage, and survival at 1 day post hatch) (Bennetau-Pelissero et al., 2001).

Results indicated growth was not affected by dietary genistein and plasma cholesterol levels were not different among treatment groups (Bennetau-Pelissero et al., 2001). For male Rainbow Trout, dietary genistein increased plasma VTG levels above controls, except during spawning, while lower plasma T levels occurred for genistein-fed trout, except during spermatogenesis (Bennetau-Pelissero et al., 2001). Levels of 11-KT were significantly lower in males at ten weeks post initiation and during spawning periods, where a significant dose-dependent decrease occurred with increasing dietary genistein (Bennetau-Pelissero et al., 2001). Also, gonadotropin levels remained low with no significant differences for males, but both βFSH and βLH were consistently lower in the 500 mg/kg treatment trout. A similar pattern was seen in 17α,20β$(OH)_2$-progesterone,

with significantly lower levels occurring in the 500 mg/kg treatment (Bennetau-Pelissero et al., 2001). For female trout, the following occurred: 1) no significant differences were seen in plasma E2 trends among groups, 2) plasma VTG levels were significantly higher in genistein fed treatments at early vitellogenesis and spawning, 3) plasma T levels in 1,000 mg/kg treatment were significantly lower than controls at the initiation of the test and then significantly higher during spawning, and 4) plasma 11-KT remained low and did not vary except during full vitellogenesis (Bennetau-Pelissero et al., 2001). Sperm volume showed a significant increase with increasing genistein levels, while spermatocrit significantly decreased in the 1,000 mg/kg treatment, and sperm motility significantly decreased in both genistein treatments (Bennetau-Pelissero et al., 2001). In female Rainbow Trout, the post-spawn residual gonad mass was significantly lower for trout fed the 1,000 mg/kg genistein treatment (Bennetau-Pelissero et al., 2001).

These results indicated that genistein had some effects on the reproductive performance of Rainbow Trout; however, there was no clear dose-dependent response, with the 500 mg/kg treatment seemingly more detrimental to the trout than the 1,000 mg/kg treatment (Bennetau-Pelissero et al., 2001). The authors suggested that the dual role of genistein, as an *in vivo* agonist and antagonist of E2, helps explain the paradoxical dosage effects on the estrogen functions related to the tissue ratio of endogenous estrogens to genistein (Bennetau-Pelissero et al., 2001). The overall effect of genistein on trout reproduction resulted in delayed reproduction in females and acceleration in males, with gonadal development greatly dependent upon the reproductive stage and endogenous estrogen secretion (Bennetau-Pelissero et al., 2001).

To determine differences in ER responses between salmonid species, Tollefsen et al. (2002) studied the Rainbow Trout and Atlantic Salmon (*Salmo salar*) ER response to a variety of estrogenic compounds, including genistein. The authors performed *in vitro* saturation and competition studies using hepatic ER's from liver tissues collected from each sex of both species, which were exposed to test compounds at varying concentrations (Tollefsen et al., 2002). Results showed that genistein was effective in displacing E2 from binding sites for both species with a potency 30-200 times less than 17β-E2 (Tollefsen et al., 2002). Equilibrium inhibitory concentrations (i.e., concentration where 50% of E2 binding is inhibited) of genistein in Rainbow Trout and Atlantic Salmon were 2.1×10^{-7} and 3.9×10^{-7}, respectively (Tollefsen et al., 2002). Likewise, relative binding affinities (i.e., equilibrium inhibitory concentration relative to concentration of E2) of genistein in Rainbow Trout and Atlantic Salmon were 3.6 and 0.48, respectively (Tollefsen et al., 2002). The authors concluded that ER's in both species had similar affinity and specificity when binding natural steroids to estrogen mimics displaying similar ER binding requirements (Tollefsen et al., 2002).

Latonnelle, Fostier, et al. (2002) conducted a study to determine the ER affinities of several different phytoestrogens in Rainbow Trout. The authors removed and prepared livers for an ER-binding assay measuring total, non-specific, and specific binding; the assay used concentrations of a radio-inert compound competing for half of the bound [^{3}H]-E2, which were designated as DC_{50} values (Latonnelle, Fostier, et al., 2002). Results indicated that two groups formed, based on DC_{50} values, with genistein (570 nM), coumestrol (400 nM), and formononetin (260 nM) composing one group and equol (5,300 nM), daidzein (9,050 nM), and biochanin A (>100,000 nM) composing the second

group. Based on these values, the relative affinity was calculated as 100*(DC_{50} E2/DC_{50} competitor) for genistein, coumestrol, and formononetin resulting in relative affinities of 1.22, 1.75, and 2.69, respectively, with an average affinity 60 times lower than E2, while equol, daidzein, and biochanin A had relative affinities of 0.13, 0.077, and <0.007, respectively, resulting in affinities 10-400 times lower than E2 (Latonnelle, Fostier, et al., 2002). The authors concluded that phytoestrogens can affect the endocrine system through competition for ER sites, redirecting pathways of natural estrogens and estrogenic activity (Latonnelle, Fostier, et al., 2002).

In a separate study, Latonnelle, LeMann, et al. (2002) compared the differences and discrepancies of the physiological effects of phytoestrogens between data obtained from *in vivo* and *in vitro* methods. As in their previous *in vitro* study, the authors removed liver tissue, prepared hepatocyte cultures, exposed them to natural steroid and phytoestrogen compounds and analyzed the corresponding VTG expression using ELISA (Latonnelle, Le Menn, et al., 2002). For the *in vivo* study, Rainbow Trout (mean weight = 350 g) were fed one of four diets containing 0.0, 0.2, 2.0, and 20 mg genistein/L for 8 weeks (Latonnelle, Le Menn, et al., 2002). Blood samples were collected every 2 weeks and analyzed with the same ELISA methods as done with the *in vitro* samples to determine VTG expression (Latonnelle, Le Menn, et al., 2002). Results indicated that no significant difference in VTG level occurred in Rainbow Trout fed genistein-enriched diets (Latonnelle, Le Menn, et al., 2002), which corresponds with previously published data indicating that 1,000 mg genistein/L is needed to induce vitellogenesis in this species (Bennetau-Pelissero et al., 2001). Also, comparisons of the estrogenic effects of E2, genistein, and formononetin from the *in vitro* study showed genistein and formononetin

to be less active than E2 by 20-100 and 60-300 times, respectively (Latonnelle, Le Menn, et al., 2002).

In a slightly different approach to genistein research in aquaculture diets, D'Souza et al. (2005) studied the deposition on genistein into muscle tissues and any effects upon the physical, chemical, and sensory quality of Rainbow Trout. Rainbow Trout fry (mean weight = 4.5 g) were fed one of four experimental diets containing 0, 500, 1,000, or 3,000 mg genistein/kg diet for a 12-month period with fish sampled for individual average weight and to obtain fillets at middle (six month) and end of study (D'Souza et al., 2005). All fillets were analyzed for proximate composition, color, thiobarbituric acid reactive substance (TBARS), and genistein levels and also prepared and served to a group of panelists for sensory analysis (D'Souza et al., 2005). Analysis of genistein-spiked muscle tissues resulted in an average genistein recovery of 56.1 ± 8.3%, while statistical analysis showed no significant differences among the diets for any of the studied parameters, except for statistically reduced TBARS levels in fish fed all diets with genistein at six months and the 500 mg/kg at 12 months when compared to control fish (D'Souza et al., 2005). Likewise, the authors did find significant, positive relationships between dietary and tissue genistein levels at both sampled time intervals (D'Souza et al., 2005). Based on these results, the authors concluded that genistein deposition in Rainbow Trout muscle tissue did not adversely affect growth, proximate composition, color, or flavor, while lowered TBARS levels suggest the ability of genistein to improve shelf life during refrigerated storage (D'Souza et al., 2005).

In another physiological study on isoflavones, Ng et al. (2006) investigated the effects of genistein, daidzein, and glycitein on E2 metabolism in the kidneys and livers of

Rainbow Trout, Atlantic Salmon, and Lake Trout (*Salvelinus namaycush*). The authors used *in vitro* cultures produced from liver and kidney tissues collected from fish of each species and exposed the tissues to increasing concentrations of genistein from 0.001-10 μM in three replicate experiments (Ng et al., 2006). Additionally, Rainbow Trout tissues were exposed to 1 μM genistein, 1 μM daidzein, 1 μM glycitein, and a mixture of all three isoflavones with the ratio 1:0.8:0.2 μM of genistein:daidzein:glycitein (Ng et al., 2006). The exposed cultures were analyzed for water-soluble E2 metabolites, with all replicated experiments resulting in almost identical metabolic activities in liver and kidney tissues (Ng et al., 2006). Results from *in vitro* culture exposures indicated a dose-dependent relationship in both the liver and kidney of all three species, wherein increased genistein exposure led to greater inhibition of [^{3}H]-E2 metabolized into conjugated (i.e., water-soluble) forms (Ng et al., 2006). Conversely, exposure to the other individual isoflavones and mixtures did not affect E2 metabolism in Rainbow Trout kidney tissue, but did inhibit E2 in the liver with no significant differences between isoflavone exposures (Ng et al., 2006). From these results, the authors suggested that estrogenic activity exerted by isoflavones, in part, can be attributed to increased bioavailability of E2 from inhibition of E2-metabolizing enzymes, and may explain such estrogenic effects in tissues where isoflavones have a low ER affinity (Ng et al., 2006).

Along with the above mentioned physiological effects of genistein, Gontier-Latonnelle et al. (2007) sought to characterize the pharmacokinetics and bioavailability of genistein in Rainbow Trout plasma. Such characterization was accomplished via intravascular injections (4 mg of genistein/kg body weight) and oral ingestion (200 mg genistein/kg) of genistein administered to reproductively active female Rainbow Trout

(mean body weight = 1 kg), which were catheterized for blood collection procedures prior to exposure (Gontier-Latonnelle et al., 2007). This administration approach allowed for identification of first pass effects from intestinal, hepatic, branchial, and global sources following blood and tissue collections from sampled fish (Gontier-Latonnelle et al., 2007). Absorption of genistein appeared to be slow with a time to maximum concentration (i.e., T_{max}) of 11.66 h and was considered the time limiting factor of disposition with half-lives from intravascular and oral administration of 2.025 and 13.075 h, respectively (Gontier-Latonnelle et al., 2007). Relative bioavailability in trout appeared low at 6-13%, which appeared dependent on the metabolism and biotransformation via glucuronidation leading to the elimination of genistein (Gontier-Latonnelle et al., 2007). Likewise, observations of radioactive-labelled genistein found a very low occurrence of bioaccumulation within trout with the liver containing the greatest amount of radioactivity followed by intestinal fat (0.21%) (Gontier-Latonnelle et al., 2007). Also, muscle tissues contained a higher amount of radioactivity (0.14%); however, this level significantly decreased from 48-72 h and remained low (0.009%) thereafter (Gontier-Latonnelle et al., 2007). From these results, the authors indicated that genistein plasma levels are likely influenced by the feeding cycles utilized in culturing and that fish fed SBM-based diets do not expose consumers to genistein or its metabolites from fish fed such diets (Gontier-Latonnelle et al., 2007).

To provide some preliminary information on genetic expression of growth and nutrient storage, an *in vivo* study was performed and replicated 3 times on juvenile Rainbow Trout (5 month old; mean weight = 65.8 ± 1.8 g) using multiplex analysis on multiple growth-related and lipogenic genes (Cleveland & Manor, 2015). Fish received

intraperitoneal injections to mimic dietary (5 µg/g) or pharmaceutical (50 µg/g) levels of either the chemical carriers dimethylsulfoxide (DMSO) and vegetable oil (i.e., control), 5 µg E2/g body weight (BW), 5 µg genistein/g BW, 50 µg genistein/g, or 5 µg daidzein/g BW; white muscle and liver samples were collected 24 hr post injections (Cleveland & Manor, 2015). A genetic analysis system was used to simultaneously analyze two separate multiplexes of growth-related and lipogenic gene expressions in all tissue samples, while vitellogenin expression was measured using real-time PCR analysis (Cleveland & Manor, 2015). In general, the results indicated that genistein and daidzein produced estrogenic effects in liver tissues via up-regulation of *era1* and *vtg* gene expression with a dose-dependent response occurring for genistein (Cleveland & Manor, 2015). Likewise, the 50 µg genistein/g and 5 µg E2/g treatments affected numerous genes in similar directions and magnitudes indicating estrogen receptor-dependent regulation mechanisms as the likely cause for growth effects as evidenced in a down-regulation of the growth hormone/insulin growth factor (GH/IGF) axis seen via decreased *ghr* and increased *igfbp2b1* expressions (Cleveland & Manor, 2015). The same treatment levels also resulted in up-regulation of genes in liver tissues related to fatty acid synthesis that were directly proportional to treatment estrogenicity; thereby, further supporting estrogen receptors as mediation mechanisms and the potential for reduced growth (Cleveland & Manor, 2015). In muscle tissue samples, up-regulation of *pax7* and *myod* expressions in E2 and genistein treatments reflects the possibility of reduced growth from the inhibition of myogenesis. Also, *ghr2* expression within muscle tissue responded to E2 and genistein treatments, while *igfbp2a*, *igfbp4*, and *igfp5b1* increased expression with genistein exposure (Cleveland & Manor, 2015). Such an increase in *igfbp4* and *ifgbp5b1*

expression is indicative of a potential positive effect on myogenesis and thus growth but is inconsistent with the previously mentioned expression of the muscle regulatory factors of *pax7* and *myod* (Cleveland & Manor, 2015). The authors concluded that the studied effects of genistein and daidzein support the premise of reduced growth in juvenile trout via down-regulation of the GH/IGF axis and regulation of lipid metabolism within the liver (Cleveland & Manor, 2015). Due to the large number of genes and variations that occurred within this study, many of the findings and results were generalized for this discussion. As such, readers interested in this topic are encouraged to read this study in its entirety.

To increase the information of isoflavone effects on larval fishes, Gu et al. (2015) sought to determine the effects of dietary isoflavones on early development parameters of Atlantic Salmon larvae from exogenous feeding to the juvenile life stage. Larvae (initial mean weight = 0.17 ± 0.01 g SE) were fed a fish meal-based (negative control), SBM-based (positive control), or a fish meal-based diet with 1.5 g/kg isoflavones. Analyses included growth performance, condition, survival, digestive enzyme activities, histology of intestines (proximal and distal) and liver, and skeletal deformities (Gu et al., 2015); additional diets were created with other ANFs but they are not relevant to this manuscript and therefore, not reported. The results found very high survival (95.4-97.8%) and a normal range for the condition factor with no significant differences between treatment groups occurring for either parameters; however, FBW was significantly lower for the isoflavone diet compared to the SBM-control diet (Gu et al., 2015). For digestive enzyme activities, maltase and trypsin activities in the proximal intestine were significantly lower and higher, respectively, for the isoflavone diet compared to the fishmeal-control diet (Gu

et al., 2015). No differences were detected in the any of the proximal or distal intestinal histology parameters (i.e., mucosal fold height and fusion, lamina propria width, submucosa width, and enterocyte vacuolization and nucleus position), while liver morphology parameters indicated a significantly lower accumulation of vacuoles in larvae fed the isoflavone diet compared to both controls and diameter of hepatocytes compared to the fishmeal-control diet (Gu et al., 2015). The frequency of skeletal deformities remained low across the treatments (0.6-5.1%) with the isoflavone diet fed fish exhibiting a higher frequency of 4.3% (Gu et al., 2015). The authors concluded that the negative effects from isoflavones may have inhibited the synthesis of thyroid hormones and/or modified glycogen stores within the liver resulting in negative growth performance, digestive function, and bone formation (Gu et al., 2015).

Most studies on phytoestrogen impacts of steroidogenesis focus on reproductive steroids; therefore, Pastore et al. (2018) performed a study looking at the effects of several isoflavones on cortisol levels along with estrogenic activity, health, and growth parameters. Juvenile, grow-out stage Rainbow Trout (initial mean weight = 176.7 ± 0.7 g) were fed for 70 days one of three diets containing a proportional mixture of genistein:daidzein:glycitein (46.64%:46.65%:6.71%) at 0, 500, or 1,000 mg/kg diet resulting in dietary isoflavone concentrations of 0, 523.14, and 1,466.60 mg/kg, respectively (Pastore et al., 2018). Growth, performance, and condition metrics, similar to those mentioned in previous studies, were measured along with the following: plasma and liver VTG; plasma, muscle, and fin E2 and cortisol levels; mRNA levels of the Insulin Growth Factor I (*igf*-I), and histological analysis of gills, liver, kidney, muscle, and gut tissues (Pastore et al., 2018). The results found normal morphology of all

histologically examined tissues and no statistical differences among treatment groups for any of the studied parameters, except for a significant correlation (Spearman's correlation, $p < 0.05$; $R^2 = 0.2798$) between plasma levels of E2 and VTG densitometry values (Pastore et al., 2018). Based on these results, the authors concluded that dietary isoflavones at these inclusion levels did not induce estrogenic effects or influence reproduction, health, or growth of Rainbow Trout for this life-stage and size (Pastore et al., 2018). Furthermore, the authors indicated that the seen VTG production was most likely the cause of endogenous E2 based on the correlation of E2 to VTG (Pastore et al., 2018).

In another study, Torno et al. (2018) investigated the effects of dietary genistein on macronutrient digestibility and intestine histology of juvenile Rainbow Trout (initial weight = 144.2 ± 16.1 g). Fish were fed a control or 0.3% (i.e., 3,000 mg/kg) genistein supplemented diets containing the inert marker titanium dioxide for determination of apparent digestibility coefficients (ADCs), which were measured from stripped fecal samples collected 29.5 h post feeding on a reoccurring cycle from 11-22 day of experiment (DOE) (Torno et al., 2018). Along with ADCs, FBWs, and mortality rates, nutritional composition of fecal samples was determined and histological samples of the hindgut collected (Torno et al., 2018). No significant differences occurred for FBW, mortality, or histological differences of fish fed either diet; however, ADCs of dry matter, crude lipid, and gross energy were slightly but significantly lower for the genistein diet with the crude protein ADC for genistein diet reported as tending towards significance (i.e., *P*-value < 0.1) (Torno et al., 2018). Due to the use of equal feeding rations between diets, the authors concluded that reduced digestibility occurred solely from genistein

inclusion indicating an overall impairment of nutrient utilization; however, this impairment did not occur due to changes in intestinal morphology based on histological examination of the hindgut (Torno et al., 2018). It also should be noted that the lack of differences in growth and mortality parameters could be a result of limited time frame of the study (i.e., 22 days total), but histological changes should have occurred within the given time frame, according to the authors (Torno et al., 2018).

While a majority of the research on this topic focuses on the role of SBM and corresponding genistein levels as a fish meal replacement, Torno et al. (2019) studied a different dietary aspect by examining the use of genistein as a potential complement and/or fish oil replacement. To examine this possibility, juvenile Rainbow Trout were fed for a 62-day period one of four different diets containing 0 or 4% fish oil with or without 0.3% genistein labelled F0, F0+G, F4, or F4+G (Torno et al., 2019). Growth (FBW and specific growth rate [SGR]), performance (feed conversion ratio [FCR], protein efficiency ratio [PER], protein productive value [PPV], and daily feed intake [DFI]), and condition (Fulton condition factor [K] and hepatosomatic index [HSI]) parameters were measured and calculated along with the collection of blood and whole-body tissues for hematocrit levels and whole-body nutrient and fatty acid composition (Torno et al., 2019). Overall growth results for all fish were poor with only 41-69% weight increase and no statistical differences occurred for FBW, SGR, HSI, K, and hematocrit levels between the F0, F0+G, F4 and F4+G diets (Torno et al., 2019). In contrast, DFI was statistically lower between diets with genistein and their corresponding controls (i.e, F0 vs. F0+G and F4 vs F4+G) Likewise, PER and PPV were statistically higher for fish fed the F0+G diet compared to F0, while FCR was statistically lower (Torno et al., 2019). A

statistically higher crude ash level for fish fed the F0+G compared to the F0 diet provided the only statistically different comparison for any of the whole-body and fatty acid compositions comparisons. The authors concluded that the reduced DFI may have resulted in impairment of other growth and performance parameters; however, the increased protein utilization from genistein inclusion enhanced growth to levels similar of control fish (Torno et al., 2019). The authors also suggested that possible genistein accumulation (not measured) within the recirculating aquaculture system used for the study may have resulted in the overall poor growth seen in all treatments; thus, careful consideration on potential negative effects on cultured fish should be taken when using diets with phytoestrogens within such culture systems (Torno et al., 2019).

Studying a different aspect of isoflavones in aquaculture, Turan and Yigitarslan (2019) explored the use of soybean isoflavones to induce sex reversal in Rainbow Trout via immersion treatments. Rainbow Trout larvae (14 days post hatch [dph], mean weight = 0.123 ± 0.03 g) received a 2-h immersion treatment in 0, 0.10, or 0.20 g/L of commercial soybean isoflavone extract; immersion treatments occurred twice for 1 month (Turan & Yigitarslan, 2019). The authors made no attempt to determine isoflavone types and concentrations contained in the soybean isoflavone extract. Treated larvae were then cultured for a 4-month period to allow sufficient gonad development for histological analyses (Turan & Yigitarslan, 2019). Following this culture period, growth and survival rates were calculated and gonad tissue was collected and analyzed to determine sex ratios (Turan & Yigitarslan, 2019). Results found a significantly female-skewed sex ratio (69 female:31 male) in fish treated at the 0.20 g/L level when compared with control fish (49 female:51 male), while no intersex individuals or damage occurred in testicular or

ovarian tissues; no differences occurred in growth or survival rates (Turan & Yigitarslan, 2019). The authors concluded that this study provides the first evidence of low-level, soybean isoflavone concentrations as a feminization agent in immersion treatments for Rainbow Trout; however, further research is warranted to determine an optimal treatment regime to induce a 100% sex reversal in this species (Turan & Yigitarslan, 2019).

Channel Catfish

Another common food fish cultured in the United States is Channel Catfish. Green and Kelly (2008) studied the effects of genistein on the reproductive performance of Channel Catfish by measuring spermatozoa motility time, motility rank, ATP content, and fertilization rates. Semen was collected from seven male Channel Catfish (mean weight = 2.5 ± 0.3 kg SE) through surgical removal and malleation preparation of mature testes and eggs collected from 2 female catfish (mean weight = 3.2 ± 0.5 kg SE). A 30 mL aliquot of a phenol red-free Hanks's balanced salt solution (HBSS) was mixed with testes from each male Channel Catfish and samples were filtered through a 250 μm mesh (Green & Kelly, 2008). Sperm incubation occurred *in vitro* by adding 1 mL of sperm suspension from each Channel Catfish to 4 mL of HBSS containing one of incremental (10^{-1}) genistein concentrations from 10^{-9} to 10^{-2} molar based on serial dilutions of a stock 1 molar solution in 25 cm^2 tissue culture flasks (Green & Kelly, 2008). Spermatozoa motility time, rank, and concentration was determined by analyzing 10 μl of incubated sample activated by 1 drop of pond water, while inactivated spermatozoa were separately extracted and measured for ATP content (Green & Kelly, 2008). Channel Catfish eggs collected from two females were divided into triplicate 125 g samples and fertilized with 1 mL of sperm from 3 different males within the same treatment (3 mL of sperm

solution). Fertilization rates were determined as the ratio of total live eggs to total eggs in the sample by examining eggs 48 h after exposure to spermatozoa (Green & Kelly, 2008).

The authors found significant negative relationships between genistein concentration and spermatozoa motility time, rank, and ATP content (Green & Kelly, 2008). Similarly, fertilization was significantly dependent on *in vitro* genistein incubation concentrations, with a significant negative effect on fertilization rates but yet a significant positive relationship between ATP content and percent fertilized (Green & Kelly, 2008). The authors concluded that spermatozoa of fish exposed to genistein could have reduced quality and motility due to reductions in ATP content (Green & Kelly, 2008). Further study is warranted to examine phytoestrogen levels in broodstock feeds and culture system source water, as relatively high concentrations of phytoestrogens may negatively impact sperm function and subsequent fertilization success (Green & Kelly, 2008).

In a follow-up study, Green and Kelly (2009) investigated the effects of timing and feed duration of genistein on phenotypic sex characteristics of Channel Catfish. This was accomplished by histological examination of gonad tissue from fish after exposing them to several dietary genistein concentrations, during the period of sexual differentiation (Green & Kelly, 2009). Two feeding experiments were performed using 60-150 days post-hatch (dph) and 5-140 dph Channel Catfish, to correspond with the timing of testes formation at 90-102 dph and onset of ovarian differentiation at 19-22 dph (Green & Kelly, 2009; Patiño et al., 1996). Fifteen fish per tank, held in triplicate aquaria, were fed a commercial Rainbow Trout feed treated with 0 (control), 2, 4, or 8 mg genistein/g feed twice daily for the duration of the feeding trials (Green & Kelly, 2009). Livers were sampled to calculate HSI and measure ethoxyresorufin-O-deethylase activity

(EROD). Longitudinal saggital sections of gonads were excised, mounted to microscope slides, stained with hemotoxylin and eosin, and examined to assign gonadal sex; intersex individuals were characterized by the presence of oocytes intermingled in testicular tissue (Green & Kelly, 2009). There were no significant differences observed in HSI or EROD activity, while phenotypic sex was found to be significantly dependent on dietary genistein concentrations, with intersex individuals observed in the 4 and 8 mg genistein treatments for both feeding trials and in the 2 mg genistein treatment for the 50-140 dph feeding trial (Green & Kelly, 2009). Also, the researchers observed a paradoxical sex reversal with increased proportions of phenotypically male individuals occurring in the 4 and 8 mg genistein treatments from the chronic dietary exposure to genistein. Therefore, Green and Kelly (2009) concluded that a sex reversal occurred where increased levels of dietary genistein shifted sex ratios to increased male phenotypes. Two possible explanations were presented to explain this paradoxical sex reversal. First, as mentioned earlier, genistein can serve both as an estrogen agonist and antagonist; therefore, while genistein has been documented to produce VTG in other teleost species (Latonnelle, Fostier, et al., 2002; Pelissero et al., 1991), it may have reduced the estrogen response by blocking ER in Channel Catfish (Green & Kelly, 2009). Second, an earlier study suggested that dietary androgens were converted to compounds with estrogenic properties by aromatase enzymes within Channel Catfish resulting in female skewed populations (Goudie et al., 1983), while phytoestrogens via aromatase pathways within mammalian cell cultures have been shown to inhibit E2 production (Brooks & Thompson, 2005). Decreased E2 synthesis may have resulted from the inhibition of aromatase, leading to a higher proportion of phenotypic males (Green & Kelly, 2009).

Japanese Medaka

A study performed by Zhang et al. (2002) focused on the potential estrogenic and/or anti-estrogenic effects of genistein on the endocrine functions of Japanese Medaka (*Oryzias latipes*) in comparison to 17β-E2. Five treatment groups of 20 male and 20 female Japanese Medaka (mean weight = 0.26 g) received 5 μl intraperitoneally injections of 75, 750, or 30,000 ng genistein/fish, 300 ng 17β-E2/fish (positive control), or solvent in the carrier (negative control) (Zhang et al., 2002). Fish received these injections every 48 h for a 10 day period; followed by collection of blood, liver, and gonad samples to determine VTG production, circulating steroid concentrations, and steroidogenesis (*ex vivo*) (Zhang et al., 2002). Results for females indicated the 17β-E2 treatment significantly increased VTG in the liver compared to the negative control fish, while the 750 and 30,000 ng genistein treatments significantly increased *ex vivo* E2 release from sampled ovarian tissue compared to negative control fish (Zhang et al., 2002). Conversely, male fish in the 750 ng genistein treatment exhibited significantly lower *ex vivo* T levels than negative control fish, while circulating T levels were also significantly lower than controls for every treatment except males in the 75 ng genistein treatment (Zhang et al., 2002). Likewise, males receiving the 17β-E2 treatment had significantly higher liver VTG concentrations than the controls (Zhang et al., 2002). These results indicated that genistein produced estrogenic responses, with no evidence of any anti-estrogenic effects in Japanese Medaka (Zhang et al., 2002). However, it appeared that the estrogenic response of *ex vivo* steroid secretion was greater than that of VTG production in response to E2 injections (Zhang et al., 2002). The authors also noted the difference in sensitivity of endpoints to genistein treatments for detection of

estrogenic effects, based on the greatest magnitude of effect on *ex vivo* steroid release from genistein exposure. Therefore, *ex vivo* steroid release appeared to be more sensitive when compared to the commonly used hepatic VTG biomarker (Zhang et al., 2002).

Another study examined gonadal development in Japanese Medaka exposed to waterborne concentrations of equol and genistein (Kiparissis et al., 2003). The authors exposed newly hatched embryos to nominal concentrations of genistein at 1, 10, 100, and 1,000 μg/L and equol at 0.4 and 0.8 μg/L in static exposure systems (Kiparissis et al., 2003). When the exposed fish had reach sexually maturity (i.e., after 100 dph), phenotypic sex was determined by examining the secondary sex characteristics of urogenital papilla shape, dorsal fin shape, anal fin shape, and the presence/absence of papillary processes (Kiparissis et al., 2003). Histological preparations from sagittal sections of whole fish were evaluated for gonadal tissue condition along with gametogenesis stages (Kiparissis et al., 2003). Results from male fish exposed to genistein indicated a concentration-dependent increase of connective tissue in testes, fibrosis around testicular lobules, decreased spermatozoa densities, and a low incidence (2 fish) of intersex in the 1,000 μg/L treatment (Kiparissis et al., 2003). Similarly, poor agreement (38%) occurred between phenotypic and histological sex determination of male fish in the 1,000 μg/L treatment, along with all affected males exhibiting the phenotypic female shape of the urogenital pore (Kiparissis et al., 2003). Females also showed concentration-dependent increases in the following parameters indicating delayed oogenesis: oocyte atresia from 9-38%, reduction of oocyte numbers, ovarian lumen size from 10-27%, and incidence of ovaries containing visible primordial germ cells from 5-19% (Kiparissis et al., 2003). Compared to genistein, equol produced greater

physiological effects upon male fish, including 10-87% occurrence of intersex, in which oocytes were present throughout testicular tissue for all cases (Kiparissis et al., 2003). Likewise, a concentration dependent increase in delayed spermatogenesis, proliferation of fibrotic tissue, and density of spermatozoa decreases from 19-29% (Kiparissis et al., 2003). Females exposed to equol exhibited delayed oogenesis similar to genistein-exposed fish (Kiparissis et al., 2003). Based on these results, the authors suggested that isoflavone compounds should be considered as endocrine disrupting because of physiological impacts on gonads and secondary sex characteristics; however, further research is needed to determine if such impacts affect population-level dynamics such as reproduction and recruitment (Kiparissis et al., 2003).

A study performed by Inudo et al. (2004) sought to measure phytoestrogen (genistein and daidzein) levels in different laboratory diets and then analyze the estrogenic activity of these phytoestrogens on Japanese Medaka. The authors completed their initial objective by analyzing six diets using HPLC and gas chromatography with electron capture detection, which found that the diets contained 0.8-126.4 µg genistein/g diet and <0.8-99.1 µg daidzein/g diet (<0.8 µg/g indicates below detection limits) (Table 2-2) (Inudo et al., 2004). For the second objective, acclimated, breeding adults were selected and fed either *Artemia nauplii* (<0.8 µg genistein or daidzein/g diet), Otohime B2 (1.4 µg genistein/g, 2.0 µg daidzein/g), or a commercial diet (58.5 µg genistein/g, 37.3 µg daidzein/g) twice daily for 28 days (Table 2-2) (Inudo et al., 2004). Fertility (i.e., ratio of fertilized eggs) was calculated from daily counts of eggs spawned during the 28-day period, while total weight, length, liver, and gonads were measured/collected at the end of this period to calculate gonadosomatic index (GSI) and HSI and determine hepatic

VTG levels (Inudo et al., 2004). Results indicated no significant differences in total length, weight, GSI, HSI, daily egg counts, or fertility with all treatment groups exhibiting >80% fertility; however, male fish fed the commercial diet had significantly higher hepatic VTG production compared to the other two diets (Inudo et al., 2004). The authors also assessed the estrogenic activity for ERα and ERβ of all nine diets studied by calculating the 10% relative effective concentration (i.e., EC_{X10}) where the concentration of the test diet showed 10% of the estrogenic activity of the control diet and estrogenic activity equal to 17β-E2 (Inudo et al., 2004). For the initial six diets studied, only one showed estrogenic activities for ERα and five for ERβ, while the commercial diet fed for the second objective showed estrogenic activities for both ERα and ERβ (Table 2-2) (Inudo et al., 2004). Based on these results, the authors concluded that diets containing high levels of phytoestrogens might induce estrogenic effects, primarily via increased VTG production in males (Inudo et al., 2004) Therefore, they recommended the use of standardized, open-formula diets that are altered to reduce estrogenic substances below effective levels (Inudo et al., 2004).

Sturgeons

One of the first studies to investigate estrogenic activity of phytoestrogens in fish was performed on Siberian Sturgeon (*Acipenser baeri*) (Pelissero et al., 1991). The authors tested the estrogenic activity of formononetin, daidzein, genistein, equol, biochanin A, and coumestrol by determining induction of VTG secretion in sturgeon and comparing it 17β-E2 activity (Pelissero et al., 1991). Four Siberian Sturgeon (mean weight = 20 g; mean length = 12 cm) each were placed into eight different treatments. All fish received intraperitoneal injections of 0.5 mL solutions containing either

formononetin or biochanin A at 0.5 mg/g body weight, daidzein or genistein at 0.2 mg/g body weight, equol or coumestrol at 0.05 mg/g body weight or E2 at 0.0001 mg/g body weight (Pelissero et al., 1991). Dosages, based on previous studies, varied among the phytoestrogens and four successive injections were administered at two-day intervals over an 8-day period (Pelissero et al., 1991). Blood collection occurred on day 10 and the plasma was analyzed for VTG levels; all phytoestrogens, except formononetin, induced VTG synthesis when compared to control fish (Pelissero et al., 1991). Biochanin A, daidzein, genistein, equol, coumestrol, and E2 induced plasma VTG levels of 98 ± 14, 2 ± 0.06, 213 ±56, 8.8 ± 2.8, 272 ± 98, and 246 ± 97 μg/mL, respectively (Pelissero et al., 1991). The authors indicated that this response demonstrates that these phytoestrogens, except for formononetin, function as estrogen agonists in Siberian Sturgeon and that dietary inclusion rates must be considered when SBM is used in fish feeds (Pelissero et al., 1991). Likewise, the authors suggested the potential for environmental contamination from estrogenic compounds in fish farm effluents (Pelissero et al., 1991).

In the previously mentioned studies by Latonnelle, LeMann, et al. (2002) and Latonnelle, Fostier, et al. (2002), effects of phytoestrogens on Siberian Sturgeon were analyzed alongside results obtained for Rainbow Trout; consistent methods were used in both studies. The authors used Siberian Sturgeon, along with Rainbow Trout, to determine if species specific responses resulted from potential variations in ER affinities (Latonnelle, Fostier, et al., 2002) and if interspecific differences/discrepancies existed between *in vivo* and *in vitro* methods (Latonnelle, Le Menn, et al., 2002). Results indicated the same phytoestrogen groupings as seen in Rainbow Trout with DC_{50} values of genistein (220 nM), coumestrol (150 nM), and formononetin (1,090 nM) composing

one group and equol (8,300 nM), daidzein (83,000 nM), and biochanin A (>100,000 nM) composing the second group (Latonnelle, Fostier, et al., 2002). Based on these values, the relative affinities for genistein, coumestrol, and formononetin were 2.3, 3.5, and 0.5, respectively, resulting in an average affinity 100 times lower than E2, while equol, daidzein, and biochanin A were 0.06, 0.006, and <0.005, respectively resulting in affinities 10-600 times lower than E2 (Latonnelle, Fostier, et al., 2002). The authors concluded that phytoestrogens did affect the endocrine system through ER competition and that a higher affinity of genistein in Siberian Sturgeon compared to Rainbow Trout suggests that metabolism of this isoflavone could be species dependent and sensitive (Latonnelle, Fostier, et al., 2002).

Based on the results from Latonnelle, LeMann, et al. (2002), Siberian Sturgeon were affected by genistein, with a statistical increase in VTG production when fed the 20 mg genistein/l enriched diet during the *in vivo* study. *In vitro* study results found relative potencies of phytoestrogens when compared to E2 ranging from 1/400 to 1/10,000, with genistein, daidzein, and formononetin having relative potencies of 1/900, 1/700, and 1/500, respectively (Latonnelle, Le Menn, et al., 2002). In comparison to the Rainbow Trout results, Siberian Sturgeon appear to be ≈50 times more sensitive to genistein based on *in vivo* trials, while differences in relative potencies between the species from *in vitro* trials may be caused by species specific metabolic reactions (Latonnelle, Le Menn, et al., 2002). The authors concluded that both *in vitro* and *in vivo* approaches should be performed when studying physiological effects of estrogen mimicking compounds, as Siberian Sturgeon reactions to the studied compounds showed discrepancies in estrogen potency between these approaches (Latonnelle, Le Menn, et al., 2002).

Along with studying disposition of genistein in Rainbow Trout, Gontier-Latonnelle *et al.* (2007) also investigated the same pharmacokinetics and bioavailability in juvenile Siberian Sturgeon (mean body weight = 900 g) in an effort to explain differences in the estrogenic effects of genistein between the species. The fish underwent the same exposure methods mentioned above except that sturgeon received 4X less genistein than trout in order to mimic fish culturing practices for this species. Like trout, absorption of genistein by sturgeon was low with a T_{max} of 10.7 h with half-lives from intravascular and oral administration of 12.35 and 12.68 h, respectively (Gontier-Latonnelle et al., 2007). However, levels of genistein in sturgeon plasma samples were 10.5X higher than trout with a maximum concentration (i.e., C_{max}; 0.27 ng/mL) 22X higher than trout (0.012 ng/mL) (Gontier-Latonnelle et al., 2007). These differences can be attributed the conjugation process of sulfation in sturgeon compared to glucuronidation in trout resulting in a slower "auto-purification" capacity from a weaker elimination speed constant (6.2X lower) and lower total plasma clearance (10X lower) (Gontier-Latonnelle et al., 2007). Along with these differences, the bioavailability and corresponding elimination of genistein was also tied to the process of sulfation (i.e., transfer of sulfate containing compounds by sulfotransferases to hydroxyl or amino groups [Günal et al., 2019]) with bioavailability being 2X higher in sturgeon (18.9%) compared to trout (6-13%) resulting in less effective elimination of genistein in sturgeons (Gontier-Latonnelle et al., 2007). Genistein metabolites were detected from radioactivity levels in sturgeon plasma, which indicated that sturgeon preferentially biotransformed genistein into sulfate conjugates providing further evidence for the differences due to sulfation; low radioactivity in trout plasma prevented performance of this analysis

(Gontier-Latonnelle et al., 2007). While sturgeon maintained higher levels of plasma genistein or its metabolites, bioaccumulation did not greatly differ between the two species with radioactivity distribution between the organ samples not exceeding 0.5 and 0.9% for trout and sturgeon, respectively (Gontier-Latonnelle et al., 2007). Similar to trout, the highest radioactivity was observed in sturgeon liver followed by muscle tissue (0.14%); however, sturgeon contained 10X more radioactivity in the posterior kidney (0.12%) and gills (0.11%) (Gontier-Latonnelle et al., 2007). Also, sturgeon muscle tissues contained a higher amount of radioactivity (0.14%) similar to trout, which in similar fashion, significantly decreased from 48-72 h and remained low (0.007%) thereafter (Gontier-Latonnelle et al., 2007). While not an exhaustive explanation of the species sensitivities to genistein, the authors concluded that sturgeon maintain more efficient absorption and weaker elimination of genistein when fed lower ingestion rates (Gontier-Latonnelle et al., 2007). However, the authors also indicated that genistein levels present from SBM-diet fed, cultured sturgeon, they do not pose a significant consumption risk to humans (Gontier-Latonnelle et al., 2007).

Due to its long reproductive cycle affecting the amount of produced caviar, a study was performed to increase oocyte development in female Beluga Sturgeon (*Huso huso*) using genistein, while also looking at growth, condition, performance, and other reproductive effects (Jourdehi et al., 2014). Female Beluga Sturgeon (five years old; mean weight = 13.25 ± 0.3 kg; total length = 140.3 ± 1.2 cm) were fed increasing levels of genistein at 0, 0.2, 0.4, 0.8, and 1.6 g/kg diet over a one-year period (Jourdehi et al., 2014). Parameters analyzed included growth (growth rate, FBW and SGR), condition (K), feeding performance (FCR and PER), oocyte diameter, and ovary histology, along

with steroid hormones (T, 17β-E2, and 17α-hydroxy progesterone), alkaline phosphatase (ALP), calcium, phosphorus, and plasma cholesterol from blood samples (Jourdehi et al., 2014). Overall growth increased (13.25 ± 0.3 kg to 29.26 ± 0.6 kg) in all the treatments; however, no significant differences where seen in any growth or feeding performance parameters among treatments (Jourdehi et al., 2014). Similarly, no differences were seen in oocyte diameters, sex steroids, or ALP compared to the control diet at the end of the study; however, calcium and phosphorus levels were significantly higher in fish fed the 1.6 g/kg diet compared to the control, along with cholesterol levels in the 0.8 and 1.6 g/kg treatments (Jourdehi et al., 2014). From these results, genistein did not promote any overall positive effects on growth or oocyte development. However, increased calcium and phosphorus levels due suggest increased reproductive performance as both ions are used as major minerals within maturing eggs during vitellogenesis (Jourdehi et al., 2014).

In a more recent study on Russian Sturgeon (*Acipenser gueldenstaedtii*), sex-related genetic factors were analyzed to evaluate the effects of genistein, daidzein, and coumestrol on the expression of these factors (Fajkowska et al., 2021). The researchers fed juvenile Russian Sturgeon (100 dph) one of four diets containing either 500 mg genistein/kg feed, 10,000 mg daidzein/kg, 10 mg coumestrol/kg or a control diet with no dietary phytoestrogens and determined that individual fish received an average of 0.23g genistein, 4.92g daidzein, and 0.005g coumestrol from 100 to 365 dph (Fajkowska et al., 2021). After 265 days of feeding, 30 fish per treatment were euthanized and processed using standard histological methods to determine sex and potential histomorphological changes (Fajkowska et al., 2021). Additionally, 100 mg of gonad, liver, and brain tissue each was collected and analyzed to determine the sex-related gene expression of the

amh, *ar*, *cyp19*, *dmrt1*, *erα*, *erβ*, *foxl2*, *sox9*, *star*, *vasa*, and *vtg* genes for these individuals (Fajkowska et al., 2021). Histological analyses revealed all treatments, including the control, contained 3-9 individuals with intersex characteristics, while no male fish were observed in the genistein and coumestrol treatment groups (Table 2-3) (Fajkowska et al., 2021). In gonad tissue, *dmrt1*, *sox9*, *amh*, *ar*, *foxl2*, *cyp19*, and *erα* showed the highest expression among all genes studied, with females having statistically higher expressions of *dmrt1*, *sox9*, *amh*, *foxl2*, *cyp19*, *erα*, *erβ*, and *vtg* genes in the control group compared to all treatments (Fajkowska et al., 2021). For brain tissue, all genes were expressed with *amh*, *foxl2*, *erα*, *erβ*, and *star* being statistically higher and *dmrt1*, *ar*, and *vtg* statistically lower in the control group compared to all treatments for females (Fajkowska et al., 2021). Similarly, males had statistically higher *dmrt1*, *amh*, *foxl2*, *erα*, *erβ*, and *star* gene expression in the control group compared to the daidzein treatment in brain tissue (Fajkowska et al., 2021). Liver tissue in males, females, and intersex individuals expressed the genes *dmrt1*, *sox9*, *amh*, *ar*, *erα*, *erβ*, and *vtg* with all sexes having higher expression in the daidzein treatment compared to the other treatments, while *fox12*, *cyp19*, *star*, and *vasa* transcripts were not seen (Fajkowska et al., 2021). From these results, the authors concluded that phytoestrogens appear to affect sex-related gene expression during gonad development primarily through effects on key gene factors and expression in brain tissue responsible for regulating sexual maintenance (Fajkowska et al., 2021). Likewise, observed down-regulation of sex-related gene expression in gonad tissue of fish in the phytoestrogen treatments indicates an inhibition of molecular pathways responsible for sexual differentiation and thus, may have contributed to the apparent female-skewed sex ratio (Fajkowska et al., 2021). The results

also indicated that dietary exposure of genistein and coumestrol may result in feminization of gonad tissue in Russian Sturgeon due to no males seen in these treatments, female-skewed sex ratios, and the presence of intersex individuals; however, additional study on this subject is warranted as the control treatment experienced *vtg* up-regulation, slightly female-skewed sex ratios, and several intersex individuals as well (Fajkowska et al., 2021).

Nile Tilapia

In one of the few studies looking at the effects of genistein on larval finfish, Akinwande *et al.* (2011) sought to use dietary genistein as an alternative approach to steroid hormones for sex reversal culture practices. To accomplish this, the authors fed 3 dph Nile Tilapia (*Oreochromis niloticus*) fry (initial weight = 6-8 mg) an experimental diet containing 500 mg genistein/kg diet or a control over an 8-week period and evaluated growth performance (i.e., SGR, and weight gain), survival, and sex ratios (Akinwande et al., 2011). Results indicated no significant changes in growth performance, survival (mean rate = 66% for both treatments), or sex ratios; however, a high number of males was observed in the genistein diet (Akinwande et al., 2011). Based on these results, the authors suggested increasing dietary genistein levels as there may be a dose-dependent response on sex ratios (Akinwande et al., 2011).

In a study performed by El-Sayed et al. (2012), two consecutive experiments were conducted to investigate the potential sex reversal of Nile Tilapia by phytoestrogens contained in SBM used in culture diets. In the first experiment, the authors fed first-feeding, Nile Tilapia larvae (mean weight = 0.01 g/fish) one of four diets containing FM or SBM with each of these diets having either 0 or 60 mg MT/kg; however, no analysis

was performed to determine the types and/or levels of phytoestrogens contained within the SBM (El-Sayed et al., 2012). Therefore, no further information from this first experiment will be presented here. In the second experiment, Nile Tilapia larvae (mean weight = 0.01 g/fish) were fed one of eight experimental diets containing 0 or 60 mg MT/kg diet and increasing levels (10, 20, and 30 mg phytoestrogen/kg diet) of genistein or daidzein for 28 days (Table 2-4) (El-Sayed et al., 2012). After the initial 28 days, fish were fed a hormone-free diet an additional 8 weeks prior to removal and examination of gonads for sex determination and survival (El-Sayed et al., 2012). Inclusion of phytoestrogens and MT in diets resulted in a significantly increased percentage of females compared to the control with a higher percent of females occurring with increasing phytoestrogen inclusion (Table 2-4) (El-Sayed et al., 2012). Survival results showed significantly lower survival in fish fed the genistein and daidzein treatments compared to the control diet with decreasing survival with increased phytoestrogen inclusion (Table 2-4) (El-Sayed et al., 2012). Survival results seemed to indicate that genistein and daidzein do affect survival, which agrees with findings on Fathead Minnows from Ingham et al. (2004) (El-Sayed et al., 2012). The authors also concluded that genistein and daidzein have a significant impact on sexual differentiation of larval Nile Tilapia, which results in a reduced masculinization effect of MT (El-Sayed et al., 2012). Therefore, the authors suggested that diets containing SBM or other plant-based sources containing higher levels of phytoestrogens be avoided during the larval growth stage if sex reversal treatments are being used to produce a monosex, male population (El-Sayed et al., 2012).

While the previously mentioned studies sought to primarily determine the sex reversal possibilities of genistein on Nile Tilapia, a study by Chen et al. (2015) studied the effects of dietary genistein on the body composition and digestive enzyme activity of this species. Juvenile Nile Tilapia (initial weight = 10.47 ± 1.24 g) were fed increasing levels of genistein at 0, 30, 300, and 3,000 µg/g for an 8-week period and were analyzed for growth performance (i.e., SGR, feed intake, feed efficiency ratio, and FBW), survival, body composition (i.e., crude protein, lipid, and ash), and digestive enzymes in the intestine, stomach, liver and hepatopancreas (Chen et al., 2015). Results showed no statistical differences in any of the growth performance parameters, survival (range = 91.11-93.33%), body composition, or stomach amylase activity; however, observational decreases of 15.82 and 12.30% between the 3,000 µg/g and the control diet were noted for FBW and SGR, respectively (Chen et al., 2015). Protease and amylase activities were significantly lower in the remaining tissues of fish fed the 3,000 µg/g and control diets, while those fed the 300 µg/g diet had significantly lower stomach protease and intestine amylase activities compared to the control fish (Chen et al., 2015). The authors indicated that the genistein mechanism responsible for the reduced enzyme activities is unknown but suggested that genistein may decrease gene expression of these enzymes (Chen et al., 2015). Likewise, it was proposed that such decreases in enzyme activity were most likely the cause of the observational decreases seen in growth performance (Chen et al., 2015).

Siamese Fighting Fish (Bettas)

Clotfelter and Rodriguez (2006) studied the effects of waterborne genistein, equol, and β-sitosterol on male Siamese Fighting Fish (*Betta splendens;* i.e., Bettas) behavior, which included swimming activity, aggression at a perceived rival male, and

nest building. Male Bettas (initial mean weight = 1.55 ± 0.03g [SE]) were exposed for a 28-day period to a negative control (ethanol vehicle only) or one of the following chemical, waterborne concentrations: 17β-E2 at 10, 100, 1,000, or 10,000 ng/L; genistein at 1, 10, 100, or 1,000 µg/L; equol at 10, 100, or 1,000 µg/L; or β-sitosterol at 10, 100, or 1,000 µg/L (Clotfelter & Rodriguez, 2006). Behavioral assessments of spontaneous swimming activity and aggressive behavior (response latency time and opercular display duration) were conducted prior to and following chemical exposure, while analysis of nest building to determine parental investment and survival was only performed post-exposure (Clotfelter & Rodriguez, 2006). Results from the behavioral assessments indicated significantly reduced activity and response latency time of male fish in the 1,000 µg equol/L compared to controls, while significant dose-dependent effects where seen in opercular display durations for 17β-E2, genistein, and equol (Clotfelter & Rodriguez, 2006). No significant differences were seen in nest building activities or nest size (Clotfelter & Rodriguez, 2006). These results showed the ability of waterborne phytoestrogens to alter and suppress aggressive behavior in male Bettas and led the authors to concluded that such alterations could have potential negative population-level consequences and additional, physiological impacts on exposed fishes (Clotfelter & Rodriguez, 2006).

In another study on male Bettas, Stevenson et al. (2011) investigated the effects of short-term, waterborne exposure to genistein and β-sitosterol on gonad size, circulating steroid hormone levels, sperm concentration, sperm motility, and *in vivo* fertilization success. Sexually mature, male Bettas (about 1 year old; 0.95-2.93 g; 32.82-43.70 mm) were used in two experiments (Stevenson et al., 2011). The first experiment dosed fish at

1 µg genistein/L, 1,000 µg genistein/L, 1 µg 17β-E2/L (positive control), or with the negative control, while the second experiment used 1 µg genistein/L, 1 µg β-sitosterol/L, a mixture with 1 µg/L of both genistein and β-sitosterol, and the same positive and negative controls; both experiments were semi-static exposures lasting for 21 days (Stevenson et al., 2011). The first experiment analyzed the circulating hormones of 11-KT and E2, which were excreted into culture water after male fish were visually stimulated by a sexually receptive female conspecific (Stevenson et al., 2011). For the second experiment, gonads were removed from sample fish following the exposure period and used to analyze GSI, sperm concentration, and several sperm motility metrics including, percent motility, curvilinear velocity, straight line velocity, and smooth path velocity (Stevenson et al., 2011). Additionally, live treatment males were also used to determine fertilization rates by allowing reproductive activities with female Bettas and analyzing the corresponding eggs for fertilization (Stevenson et al., 2011). Results indicated that no significant differences occurred in 11-KT or E2 levels of dosed fish except for a significantly lower amount of 11-KT produced in the 1,000 µg genistein/L compared to 1 µg genistein/L treatment in the first experiment (Stevenson et al., 2011). For the second experiment, sperm concentrations were significantly lower in the 1 µg genistein/L treatment than the positive control and straight line velocity was significantly lower in the positive control compared to all other treatments except the 1 µg genistein/L; all other metrics were not statistically different (Stevenson et al., 2011). From these results, the authors concluded that short-term, waterborne exposure to genistein and/or β-sitosterol did not produce any noticeable endocrine disruption in adult male Bettas and suggested that future research focus on comparisons of juvenile and adult life stages

exposed to waterborne phytoestrogens for a variety of exposure durations to determine potential periods of vulnerability to endocrine disruption (Stevenson et al., 2011).

In a similar study, Brown et al. (2014) sought to determine the effects of genistein and β-sitosterol on steroid hormone levels and any subsequent behavioral and physiological changes in female Bettas. Sexually mature, female Bettas (≥1 year old; 0.37-1.46 g; 24.33-38.85 mm) were used in two experiments and exposed to test chemicals for a 21-day period (Brown et al., 2014). In the first experiment, fish were dosed with 1 µg genistein/L, 1,000 µg genistein/L, 1 µg β-sitosterol/L, 1,000 µg β-sitosterol/L, or a negative control, while the second experiment exposed fish to 100 µg 17β-E2/L, 1 µg genistein/L, 1 µg β-sitosterol/L, a mixture with 1 µg/L of both genistein and β-sitosterol, or a negative control (Brown et al., 2014). The first experiment analyzed behavioral activities (i.e., response latency time, opercular display duration, and fin [dorsal and caudal] display duration) and circulating hormones of T and E2, which were excreted into culture water after female fish were visually stimulated by a male conspecific (Brown et al., 2014). In the second experiment, female fish were weighed and sacrificed to obtain gonad weights and tissues to determine GSI and histological data on oocyte maturation (Brown et al., 2014). The first experiment showed a significant reduction in opercular display duration of female fish in the 1,000 µg β-sitosterol/L treatment compared to negative control fish and no differences in any of the other behavioral or hormone metrics (Brown et al., 2014). Similarly, the second experiment found no differences between GSI or oocyte maturation for any of the treatments (Brown et al., 2014). The authors indicated that the results from this study directly contrast those

of previous work with male Bettas (discussed above) and that female Bettas are less affected by phytoestrogen exposure (Brown et al., 2014).

Goldfish

Ishibashi et al. (2002) analyzed the isoflavone contents in several diets fed to Goldfish (*Carassius auratus*) to determine the effects of phytoestrogens in fish diets and to develop standard testing protocols. Three diets, including a commercial trout feed, ornamental carp feed, and a control diet (0% SBM), were fed to male Goldfish to determine the extent of estrogenic activity. The two commercial feeds contained 47,680-390,800 ng genistein/g diet, 41,120-416,800 ng daidzein/g diet, 226-1,325 ng coumestrol/g diet, and 6.4-117 ng equol/g diet, while the reference diet contained 93.2, 130, 8.8, and 1,027 ng/g diet of genistein, daidzein, coumestrol, and equol, respectively (Ishibashi et al., 2002). Male Goldfish (initial weight = 6.4-13.4 g) were divided into three groups and fed 1.0% body weight of diet every 2 days for 31 days; blood samples along with body, testes, and hepatopancreas weights were taken at the end of the feeding trial (Ishibashi et al., 2002). Total body weight, GSI, HSI, and plasma VTG levels were analyzed from the Goldfish samples, while genistein, daidzein, coumestrol, and equol were extracted from the diets fed and analyzed using liquid chromatography-mass spectroscopy/mass spectroscopy (Ishibashi et al., 2002). No significant differences occurred in FBW, GSI, or HSI; however, plasma VTG levels were significantly higher in Goldfish fed the carp feed, while plasma VTG levels were below detectable limits for the control diet (Ishibashi et al., 2002).

An initial study by Bagheri et al. (2013) found that Goldfish fed increasing levels of SBM (35, 65, and 100%) resulted in decreased T and increased E2 levels in plasma

along with reduced eggs spawned and sperm quality. The authors suggested that phytoestrogens within the SBM most likely resulted in the reproduction impairments along with biosynthesis disruption of sex hormones (Bagheri et al., 2013); however, phytoestrogens types and levels were not measured or reported. Therefore, a follow-up study was performed investigating the effects of long-term exposure to dietary genistein and daidzein on the reproductive system of Goldfish via steroidogenesis (Bagheri et al., 2014). Larval Goldfish (20 week post hatch; mean weight = 2 g) were fed one of four diets containing 0:0 (Control), 24.263:21.702 (D1), 51.566:46.126 (D2), or 75.833:67.821 (D3) genistein:daidzein (μg/g) for a 13-month period (Bagheri et al., 2014). Body and gonad weights along with blood samples were collected monthly to obtain growth, GSI values, and circulating T and E2 concentrations from male and female fish (Bagheri et al., 2014). No statistical differences were seen in mortality for males or females, while D3 fish had significantly higher FBWs and statistically lower GSI values in November and January for both sexes compared to all other groups (Bagheri et al., 2014). For males no statistical differences were detected in E2 or T among diets at all sampled intervals; however, T levels of D3 fish were lower at all sample periods and showed a time-related decrease while E2 levels for the same group had a significant dose-dependent increase (Bagheri et al., 2014). Similar steroid patterns occurred for females; however, E2 levels dipped below those of males at the end of the feeding trial, while T was lower in all treatments in January with fish in D3 having the statistically lowest T levels (Bagheri et al., 2014). Both males and females had statistically higher E2/T ratios for the D3 treatment compared to all other treatments and across all sampled time periods (Bagheri et al., 2014). From these results, the authors

concluded that genistein and daidzein inclusion had a growth-promoting effect along with dose- and time-related effects on gonad growth (Bagheri et al., 2014). Likewise, the statistical and observational E2 and T levels suggested that the included isoflavones did affect sex steroidogenesis with potential impacts on the E2 biosynthetic pathways of this species (Bagheri et al., 2014).

Other Fish Species

A study performed by Ko et al. (1999) sought to determine if genistein produced any estrogen-mimicking activity on growth and reproduction of Yellow Perch (*Perca flavescens*). The authors fed fingerling Yellow Perch (initial weight = 13-15.5 g) one of three experimental diets twice daily for 63 days; one diet contained an E2 concentration of 10 μg/g diet (E10) and the other two had genistein concentrations of 0.75 (G0.75) and 7.5 (G7.5) mg/g diet (Ko et al., 1999). Blood and subsequent plasma samples were collected along with gonad samples for analyses (Ko et al., 1999). Results indicated growth in length and GSI values of Yellow Perch receiving genistein treatments were not different from either the control or E10 treatment, except for females fed the G7.5 diet had less length growth than control perch and lower GSI values for both sexes fed the E10 diet compared to the other diets (Ko et al., 1999). Weight gains for both sexes fed the G0.75 diet were relatively higher than that of the controls but not statistically different, while females fed the G7.5 diet were statistically lower and males statistically similar to control fish (Ko et al., 1999). Similarly, plasma concentrations of E2 and alkali-labile phosphor-protein (ALPP) of perch receiving genistein treatments were not different from control fish, but Yellow Perch fed the E10 diet did have higher plasma ALPP compared to control fish (Ko et al., 1999). The authors indicated that the growth data suggests, but

does not prove, the ability of genistein to maintain or improve Yellow Perch growth at lower genistein inclusion levels and that plasma levels were not higher for treated groups, except for serum ALPP concentrations (Ko et al., 1999). Several possible explanations for these conclusions could be explained by utilizing "correct" levels of genistein in the diets, which have yet to be determined empirically. First, a previous study found that E2 hormone treatments promoted growth via increased food consumption (Malison et al., 1985). Therefore, reduced growth rates of female perch in the G7.5 treatment suggests that the optimal concentration to produce increased growth may be somewhat higher or lower than 0.75 mg/g level, or that a pretreatment with E2 may be required to stimulate growth of E2 target cells prior to subsequent genistein treatments (Ko et al., 1999). Second, the failure to induce vitellogenesis in perch with 0.75 or 7.5 mg/g suggests that the absorption or metabolism of genistein of perch may differ from other species, or that these levels were too low to induce vitellogenesis (Ko et al., 1999). The previously mentioned study on Siberian Sturgeon given intraperitoneal injections of genistein found that the potency of E2 necessary to produce vitellogenesis was 2,300 times greater than genistein (Pelissero et al., 1991); therefore, if the same potency held true for perch, approximately 20 mg genistein/g of diet would be necessary to induce vitellogenesis (Ko et al., 1999).

In an effort to investigate the effects of genistein on sexual differentiation, a study was performed on Striped Bass (*Morone saxatilis*) to determine its effects as an exogenous estrogen mimic found naturally in aquaculture diets (Pollack et al., 2003). Two feeding trials were performed where Striped Bass fingerlings (about 120 and 60-100 dph) were fed one of four different diets with 0 (control), 2, 4, and 8 mg genistein/g diet

for a 6-week period with the second study having an additional 6-month period of feeding on the control diet to increase overall growth for sampling purposes (Pollack et al., 2003). At the end of both studies total length and body weight measurements were obtained from sampled fish along with blood, gonad, and liver samples, which showed significant body weight increase (i.e., indication of dietary uptake by fish) (Pollack et al., 2003). The GSI and HSI were calculated from gonad tissues also taken for histological procedures, while blood samples were analyzed for plasma VTG levels (Pollack et al., 2003). Results from the first feeding trial showed the following: 1) no statistical differences in body weight gain, GSI, or HSI between control and treatment fish, 2) an average 1:1 sex ratio for all treatments based on histological analysis, and 3) significantly higher levels of VTG in the 2 and 8 mg/g treatments compared to the control and 4 mg/g diets (Pollack et al., 2003). The second feeding trial produced almost identical results with the only differences occurring from significantly reduced growth of the 4 mg/g treatment compared to the control and 8 mg/g treatments during the 6 month grow-out phase (Pollack et al., 2003). Based on these results the authors concluded that dietary genistein exposure did not greatly affect growth or reproductive development factors and that the U-shaped VTG response curve is indicative of genistein acting as both stimulatory and inhibitory mechanisms on the endocrine system (Pollack et al., 2003).

A study by Ingham et al. (2004) investigated the effects of acute exposure to genistein on survival, growth, and developmental symmetry of Fathead Minnows (*Pimephales promelas*). Juvenile Fathead Minnows (40-45 mm) were exposed to dissolved, nominal concentrations of 0 (control), 10, 20, 40, 80, 160, 320, 640, and 1,280 µg genistein/L for a 96 hr period with a 50% treatment water change occurring every 24

hr (Ingham et al., 2004). Following this exposure, the authors monitored growth and development for an 8-week period and then euthanized and measured body symmetry of the following bilateral traits: 1) post-orbital distance (i.e., eye to edge of gill cover), 2) pectoral fin length, and 3) pelvic fin length (Ingham et al., 2004). No statistical differences occurred in survival following the initial exposure or eight weeks post-exposure along with no significant dose-dependent effect on body mass; however, observational evidence showed slower growth of minnows exposed to the highest genistein concentration (Ingham et al., 2004). Similarly, no significant differences occurred in any of the symmetrical bilateral measurements, but some observational asymmetry in pectoral fin length occurred at the 320 µg/L treatment. According to the authors, these observational differences merit further investigation to determine potential negative impacts of aquatic vertebrate prey exposed to waterborne genistein from effluent outfall sources (Ingham et al., 2004).

Concurrent with Ishibashi et al. (2002) and previously described methods used for Channel Catfish, Green and Kelly (2008) examined the effects of genistein on sperm quality, motility, and fertilization rates of Walleye (*Sander vitreus*). Walleye males (mean weight = 1.31 ± 0.06 kg) were not sacrificed as milt was obtained from anesthetized fish by abdominal palpation and Walleye females (mean weight = 3.14 ± 0.16 kg) were used to obtain eggs for fertilization trials (Green & Kelly, 2008). Seminal plasma extender was used in place of HBSS to create genistein stock and serial dilution solutions. Walleye semen samples of 200 µl each were incubated in flasks containing 800 µl of seminal plasma extender containing none or one of the experimental genistein solutions (Green & Kelly, 2008). Researchers collected eggs from two female Walleye and divided eggs into

triplicate subsamples of 100 eggs per mL; egg subsamples were fertilized with 100 µl of incubated sperm solutions from 3 individual males (300 µl total) from each treatment level (Green & Kelly, 2008). Results from analyses were similar to those of obtained for Channel Catfish, except that significant ATP reductions were observed in only six out of the eight experimental treatments (10^{-7} to 10^{-2} M) and no positive relationship between ATP content and fertilization rates occurred (Green & Kelly, 2008).

To reduce the dependence on synthetic steroids for monosex induction of ornamental fish populations, Chakraborty et al. (2012) evaluated the efficacy of several non-steriodal compounds, including genistein, as alternatives for sex reversal induction of Guppies (*Poecilia reticulata*). Only information on the methods and results from the genistein treatment are presented here. Newly born Guppies were fed an experimental diet containing genistein, which had been added to a finely ground diet using a 1:1 mixture of dimethylsulfoxide:ethanol then pelletized to make a diet containing 1 g genistein/kg diet (Chakraborty et al., 2012). The fish were fed experimental diets for a 60-day period, with experimental diets fed the first thirty days and a commercial basal diet for the remaining 30 days, and then, evaluated for survival and sex determination via microscopic analysis of gonad tissue (Chakraborty et al., 2012). Unlike previously mentioned studies, intersex individuals were classified as fish maintaining female gonad structures but showing male-like coloration and gonopodium development (Chakraborty et al., 2012). Results indicated that no statistical differences occurred in survival between the treatment groups; however, the authors did indicate that genistein had the highest apparent mortality rate based on the calculated treatment means (Chakraborty et al., 2012). In contrast, a significantly higher amount of male fish (70.6%), significantly lower

amount of female fish (24.8%), and small amount of intersex individuals (4.6%) occurred in the genistein treatment compared to the controls (44.5% male, 55.5% female, 0.0% intersex) (Chakraborty et al., 2012). These results indicated a paradoxical sex reversal like the one described by Green and Kelley (2009) for Channel Catfish, which lends further evidence to the dualistic role of genistein as an estrogen agonist or antagonist as mentioned earlier. Likewise, the authors concluded that, while not as effective as 17α-MT, genistein and other non-steriodal compounds provide a possible avenue for use in sex reversal procedures but require further research to determine the optimum treatment regime needed for a 100% sex reversal in cultured Guppies (Chakraborty et al., 2012).

In order to gain preliminary information on a new aquaculture fish species, DiMaggio et al. (2016) investigated the effects of dietary genistein on sexual differentiation, growth, and survival of Southern Flounder (*Paralichthys lethostigma*). Juveniles (84 dph; weight ≈ 0.3g) were fed diets containing 0, 100, or 1,000 mg genistein/kg diet for 69 days, which was followed by an additional 132 day grow-out phase were fish were fed a genistein-free diet; length, weight, survival, and gonad tissue for sex determination were collected at the end of both periods (DiMaggio et al., 2016). Based on these metrics, results showed that sex ratios were significantly skewed to females in both genistein treatments compared to the control; however, all treatments significantly deviated from a 1:1 male:female ratio (DiMaggio et al., 2016). The high genistein treatment produced significantly lower survival rates at the end of the grow-out period and length and weight at the end of the exposure period (DiMaggio et al., 2016). From these results, the authors suggested the dualistic role of genistein as an estrogen agonist and antagonist for the skewed sex ratios; however, only comparative studies were

provided with the conclusion of that resulting androgenic/estrogenic actions involve much complexity between genistein and ER types and corresponding expression patterns, binding affinities, and coactivator proteins (DiMaggio et al., 2016). Similarly, little explanation was given for the cause of increased mortality in the high treatment except for potential cytotoxicity of cells and/or immunosuppression. Results also showed decreased growth after exposure to higher levels of genistein at the end of the grow-out period, which the authors suggested may have occurred due to mortality of small fish in the high exposure treatment (DiMaggio et al., 2016). Likewise, species-specific, sexually dimorphic growth rates in addition to the additive androgenic/estrogenic responses may have obfuscated the growth of juvenile Southern Flounder resulting in the differing growth patterns between time periods (DiMaggio et al., 2016). The authors concluded that dietary genistein was effective at feminizing juvenile Southern Flounder but decreased growth at 1,000 mg genistein/kg; therefore, until further research is performed on this species, the authors suggested that 100 mg genistein/kg should be used for the production of female, monosex populations (DiMaggio et al., 2016).

Conclusion

As the upward trend continues for aquaculture food-fish production, the demand for commercially produced and cost-effective aquafeeds will also continue to rise. These trends point to an ever-growing reliance and need for more sustainable alternatives such as plant-based feed ingredients, with SBM as one of the most suitable and available sources. As a result, a variety of studies have been undertaken to identify and understand potential anti-nutritional factors and their effects, including phytoestrogens, and their potential growth and physiological effects on fed finfish populations. Likewise,

additional studies indicated how phytoestrogen levels within soybeans can vary on a variety of factors from growth to processing procedures. However, there remains a large void of information on the effects of aquafeed processing effects on the types and levels of phytoestrogens found in the final feed product. Phytoestrogen analysis of seven different types of commercial and specialty SBMs found limited differences in the commercially produced SBMs but much higher variation in specialty SBMs. Therefore, further research is warranted to understand processing effects on SBM to determine which SBM products that are better suited to the needs of aquafeed manufacturers and aquaculture producers. Similarly, to my knowledge, no studies have looked at the potential discharge of isoflavones to natural ecosystems from SBM sources used in aquaculture settings. Several studies have looked at the introduction and fate of various phytoestrogens in aquatic environments including rivers (Kawanishi et al., 2004), streams (Kolpin et al., 2010), wastewater effluents (Liu et al., 2010), and sediments (Kelly et al., 2015) along with potential effects on aquatic organisms (Kelly et al., 2014, 2015; Rearick et al., 2014). It would be highly unlikely that all phytoestrogens present in SBM aquaculture diets would be incorporated into the cultured fish tissue; therefore, it becomes important to determine the fate of these un-incorporated isoflavones (e.g., fecal matter, feed leaching, and waste feed) within aquaculture settings and their potential pathway(s) to natural ecosystems.

While it is apparent that phytoestrogens can produce estrogenic responses in finfishes, no clear relationship occurred across or among species, with specific phytoestrogens, or dietary inclusion levels. This fact becomes apparent by the variety of *in vivo* and *in vitro* effects produced, including: ER competition and binding, skewed sex

ratio, and intersex characteristics to name a few. A large part of this variation can be accounted for by the wide range of differing study objectives and methodologies including different species, life stages, exposure pathways, phytoestrogen levels and compounds. Such variation shows that additional research is needed to help better understand the physiological mechanisms that are affected by phytoestrogens with an emphasis on developing more standardized approaches to this line of research. While definitive relationships could not be seen, it appeared that studies with larval stage or broodstock fish exhibited the most notable estrogenic responses when exposed to phytoestrogen compounds. This general response should help guide future research to focus on these life stages as they appear to be the most sensitive to phytoestrogens; thereby providing, at minimum, a starting point for more standardized testing of these compounds in aquafeed.

Table 2-1. Phytoestrogen concentrations (µg/g) of three commercially produced soybean meal proteins including solvent extracted, soy white flake, and extruded non-GMO obtained from the South Dakota Soybean Processors, Volga, South Dakota, processing plant and four specialty SBMs including Soycomil-P® from ADM Animal Nutrition, Quincy, Illinois, ME-PRO® from Prairie Aquatech, Brookings, South Dakota, and HP 300 and HP 330 from Hamlet Protein Incorporated, Findlay, Ohio.

	Phytoestrogen (µg/g)				
Soybean Meal	**Genistein**	**Daidzein**	**Equol**	**Formononentin**	**Biochanin A**
Solvent Extracted	9.33	13.3	0.0222	0.0132	0.0053
Soy White Flake	10.8	16.0	0.022	0.0176	0.006
Extruded Non-GMO	9.6	12.8	<0.004	0.0232	0.0164
Soycomil P	0.276	0.208	<0.004	<0.004	<0.004
ME-PRO	4.72	84.0	0.054	0.200	0.242
HP 300	230	591	0.0219	0.120	0.0102
HP 330	0.8	6.8	<0.004	0.120	<0.004

Table 2-2. Concentrations of total genistein and daidzein and estrogenic activities for alpha (α) and beta (β) estrogen receptors in studied diets from Inudo et al. (2004).

Diet	Genistein (µg/g)	Daidzein (µg/g)	ER[a]α		ERβ	
			EC_{X10}[b] (µg/mL)	17β-estradiol equivalent (ng/g)	EC_{X10} (µg/mL)	17β-estradiol equivalent (ng/g)
Diet 1	10.1	7.2	NA[c]	NA	1,166	34
Diet 2	13.0	8.4	NA	NA	661	56
Diet 3	11.1	6.5	NA	NA	NC[d]	NA
Diet 4	69.6	53.7	NA	NA	NC	NA
Diet 5	126.4	99.1	705	38	178	223
Diet 6	0.8	<0.8[e]	NA	NA	NA	NA
Artemia nauplii	<0.8	<0.8	NA	NA	NA	NA
Otohime B2	1.4	2.0	NA	NA	NA	NA
Commercial	58.5	37.3	1,072	36	226	214

[a] ER = estrogen receptor.
[b] EC_{X10} = 10% relative effective concentration.
[c] NA = no estrogen activity.
[d] NC = value not calculated.
[e] <0.8 = concentration below assay detection limits.

Table 2-3. Sex ratios of sampled juvenile Russian Sturgeon (*Acipenser gueldenstaedtii*) based on histomorphology analysis from Fajkowska et al. (2021).

Treatment	**Inclusion Level**	***n***	**Females**	**Males**	**Intersex**
Control	0 mg/kg diet	30	15	12	3
Daidzein	10,000 mg/kg diet	30	12	9	9
Genistein	500 mg/kg diet	30	27	0	3
Coumestrol	10 mg/kg diet	30	24	0	6

Table 2-4. Survival and sex ratios by percentage of males, females, and intersex of Nile Tilapia (*Oreochromis niloticus*) larvae fed experimental diets containing 17α-methyltestosterone (MT; 60 mg/kg diet) and increasing levels of genistein (G) and daidzein (D) from El-Sayed et al. (2012). Different letters within a column signify statistical differences at $\alpha = 0.05$.

Diet	**Phytoestrogen Level (mg/kg)**	**Male (%)**	**Female (%)**	**Intersex (%)**	**Survival Rate (%)**
FM (control)	0	49.0 *a*	51.0 *f*	0.0	90.4 *e*
FM-MT	0	97.0 *a*	3.0 *a*	0.0	77.0 *c*
FM-MT-G1	10	75.5 *b*	24.5 *b*	0.0	88.0 *e*
FM-MT-G2	20	64.5 *bc*	33.5 *bc*	2.0 *a*	82.0 *d*
FM-MT-G3	30	48.2 *c*	47.8 *c*	4.0 *a*	80.0 *cd*
FM-MT-D1	10	82.5 *c*	17.5 *b*	0.0	75.0 *c*
FM-MT-D2	20	66.0 *bc*	32.5 *bc*	2.5 *a*	56.5 *b*
FM-MT-D3	30	57.5 *c*	42.5 *c*	0.0	51.0 *a*

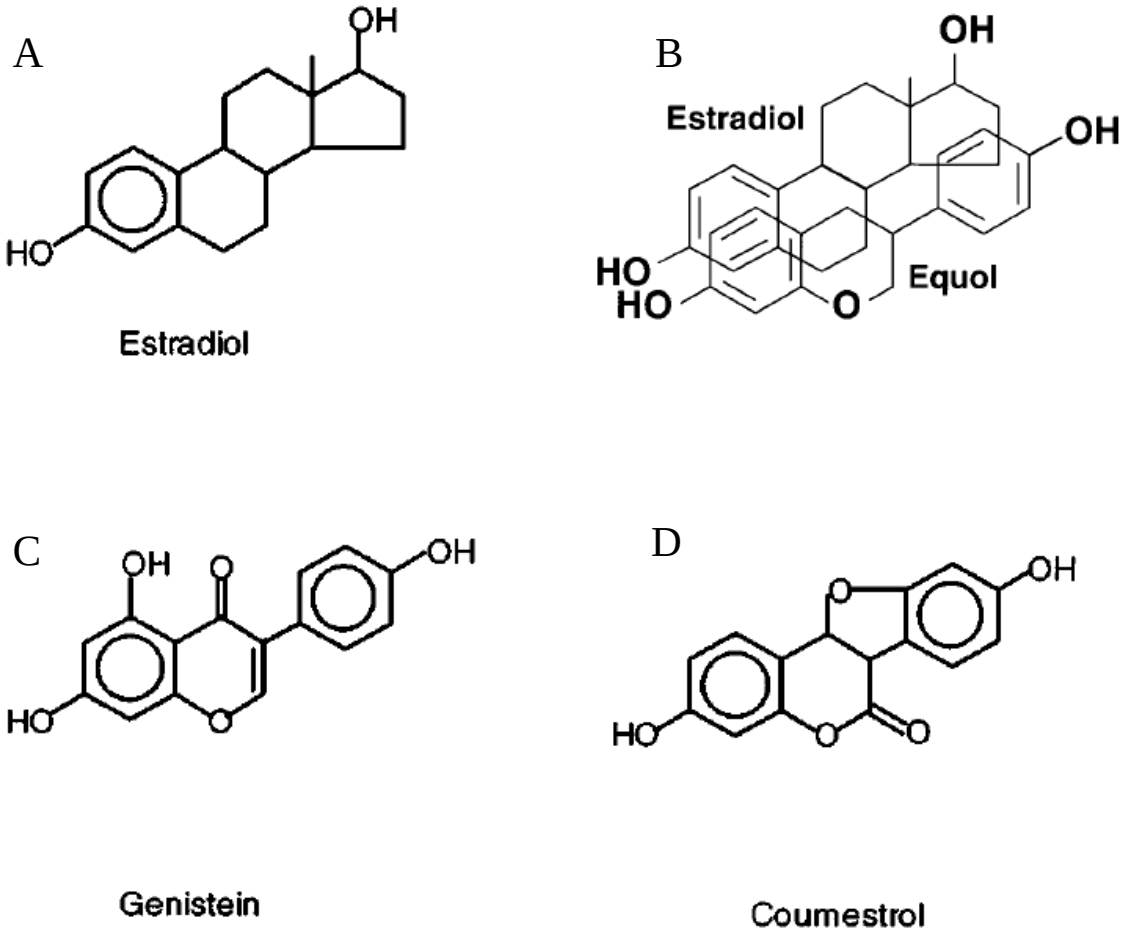

Figure 2-1. Comparison of chemical structures among 17β estradiol (A, B), phytoestrogens (genistein [C] and coumestrol [D]), and an isoflavone metabolite (equol [B]). Images modified from Kurzer & Xu (1997) and Setchell & Cassidy (1999).

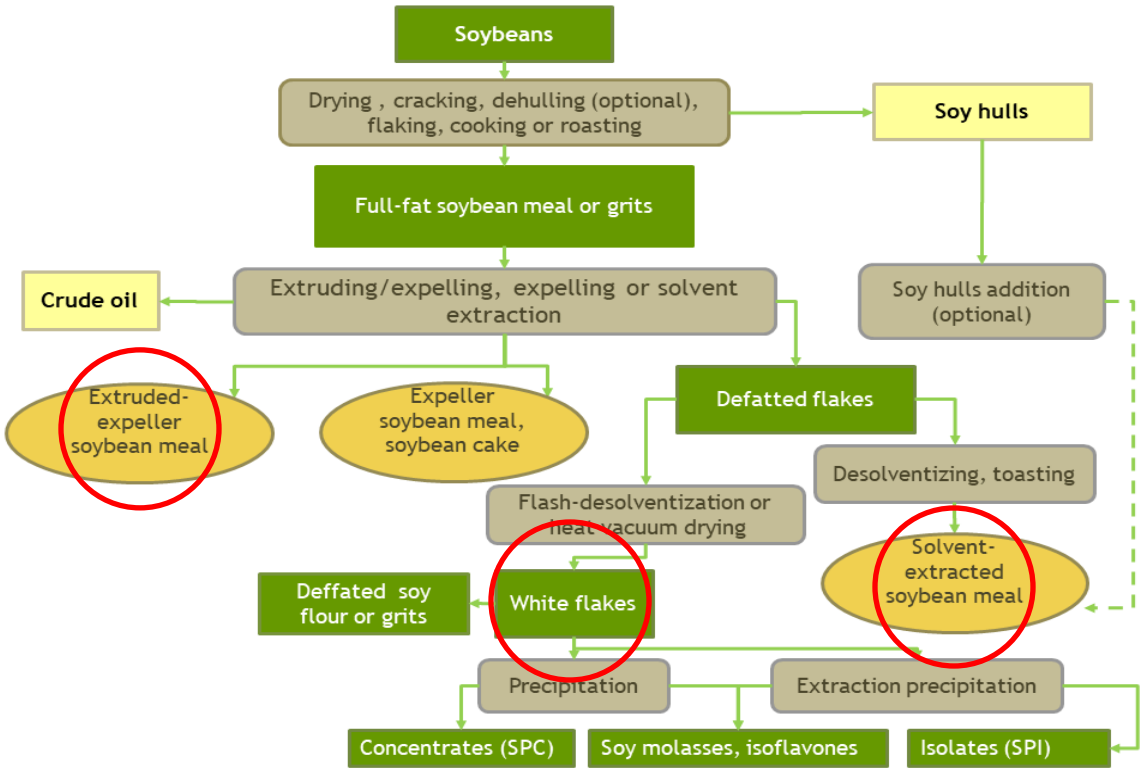

Figure 2-2. Generalized schematic of conventional soybean processing used to create different types of commercially produced soybean meals. Red circles indicate the three types of soybean meals that were collected and analyzed in this study. Figure from website https://www.feedipedia.org/content/soybean-processing. Accessed 17 April 2023.

CHAPTER 3. GROWTH, CONDITION, AND ESTROGENIC EFFECTS OF GENISTEIN IN SOYBEAN MEAL DIETS ON LARVAL RAINBOW TROUT

Introduction

Aquaculture production of food fishes is reported as one of the fastest growing food animal production sectors and has seen continued annual growth over the past seven decades (FAO, 2022). Part of this growth has been driven by the development of culture technologies and utilization of formulated aquaculture diets within the industry (FAO, 2018, 2022). A primary goal is to provide the highest quality feed ingredients for optimization of fish growth, health, and reproductive processes, while simultaneously reducing feed manufacturing costs (Pollack et al., 2003). Marine fish meal is a high quality protein ingredient that has been a staple for the aquaculture industry due to its nutritional and digestibility parameters (Abdelghany, 2003; FAO, 2018; Lech & Reigh, 2012), but increased costs associated with high demand and variable supply, from over-fished marine stocks has resulted in the research and use of plant-based protein sources (Bagheri et al., 2013; Coyle et al., 2004; Fontaínhas-Fernandes et al., 1999; Pollack et al., 2003). One such plant-based protein that has received extensive research and use is soybean meal (SBM), which has been shown to effectively replace 80% of fish meal in diets with modified SBM (Voorhees et al., 2019). This increased use is driven by the high crude protein content, amino acid profile, digestibility, lower price, global availability, and increased production of SBM (Bagheri et al., 2013; Hertrampf & Piedad-Pascual, 2000; USDA, 2023a, 2023b), but its use is dependent on SBM sources, processing methods, fish species, size/life-stage, and culture systems (El-Sayed et al., 2012). However, SBM does contain several anti-nutritional factors (ANFs) including: protease

inhibitors, lectins, antigenic proteins, phenolic compounds, oligosaccharides, phytates, and anti-vitamins (El-Sayed et al., 2012; Francis et al., 2001; Liener, 1989). In order to minimize the negative effects of ANFs, various pretreatment and processing methods can be used, including: extrusion/heat (Barrows et al., 2007; Romarheim et al., 2005), fermentation (Refstie et al., 2005; Seong et al., 2018; Yamamoto et al., 2012), and enzyme additions (Greiling et al., 2019; Kumar et al., 2020).

Along with the aforementioned ANFs, soybeans and soybean co-products (i.e., SBM, soy protein concentrate, etc.) contain phytoestrogens, which are natural estrogenic compounds found in plants and are broadly classified by their chemical structures as isoflavones, coumestans, and lignans (Francis et al., 2001; Kurzer & Xu, 1997). Of these, isoflavones and coumestans have been shown to interact with endogenous estrogen signaling pathways (Sirotkin & Harrath, 2014). The isoflavones, genistein and daidzein, account for the highest concentrations in soybeans and associated co-products and are the most biologically active of the isoflavones (Křížová et al., 2019). Experimental Rainbow Trout (*Oncorhynchus mykiss*) diets have been shown to contain 5,903 mg genistein/kg diet and 1,990 mg daidzein/kg diet from a soy protein concentrate (Mambrini et al., 1999). Because of their similar chemical structure with 17β-estradiol (E2), isoflavones can bind with both alpha and beta estrogen receptors (ER) indicating their role as estrogen agonists (Barnes et al., 2000). However, isoflavones also appear to antagonize estrogen action and effects, which can be dependent on numerous factors including isoflavones levels, available ER, and endogenous E2 (Setchell & Cassidy, 1999).

When considered as an aquaculture diet ingredient, the use of SBM has not been universally adopted due to concerns over the effects that isoflavones and other

biologically active compounds may have on growth, condition, and estrogenic responses (Bagheri et al., 2013; Ng et al., 2006). Negative effects on growth and/or condition have been reported for Yellow Perch (*Perca flavescens*) (Ko et al., 1999), Striped Bass (*Morone saxatilis*) (Pollack et al., 2003), Nile Tilapia (*Oreochromis niloticus*) (Chen et al., 2015), and Atlantic Salmon (*Salmo salar*) (Gu et al., 2015) when exposed to dietary genistein. However, other species such as Rainbow Trout (Bennetau-Pelissero et al., 2001; Torno et al., 2019), Japanese Medaka (*Oryzias latipes*) (Inudo et al., 2004), and Goldfish (*Carassius auratus*) (Ishibashi et al., 2002) showed no negative effects when exposed to dietary genistein.

One of the response metrics used to indicate exposure to estrogenic substances is the production of vitellogenin (VTG). Vitellogenin is an estrogen-induced lipoprotein produced from liver hepatocytes and is the precursor to egg yolk protein and has been reported as an excellent biomarker for exposure to estrogenic substances for both field and laboratory studies (Pait & Nelson, 2002; Pollack et al., 2003). This fact is especially true for male (mature and immature) and immature female fish due to their low levels of circulating plasma VTG as compared to the high levels seen in sexually mature female fish under normal conditions (i.e., no abnormal chemical, physiological, and/or environmental factors affecting fish) (Ishibashi et al., 2002; Pollack et al., 2003). Several studies have shown the ability of SBM-based diets containing genistein and daidzein to induce or increase VTG production in Rainbow Trout (Bennetau-Pelissero et al., 2001), Siberian Sturgeon (*Acipenser baeri*) (Pelissero et al., 1991), Striped Bass (Pollack et al., 2003), Japanese Medaka (Inudo et al., 2004), and Goldfish (Ishibashi et al., 2002). In contrast, several studies have shown conflicting results with no change to the induction of

vitellogenesis from genistein exposure in Rainbow Trout (Kaushik et al., 1995; Latonnelle, Le Menn, et al., 2002), which may have occurred from the use of lower exposure levels.

These studies suggest that the effects of isoflavones on growth, condition, and estrogenic responses can vary in magnitude due to species-specific effects and/or isoflavone exposure levels. Also, while several studies have investigated the effects of genistein on juvenile to adult Rainbow Trout, limited information exists in the literature on the potential effects of genistein to larval salmonids with no studies on larval Rainbow Trout. This lack of information is interesting because of the complex processes involved with digestive ontogeny (Rønnestad et al., 2013; Sala et al., 2005) and gonadal development/sexual differentiation (Billard, 1992) that occurs from larval to juvenile stages, which could be greatly affected by isoflavone exposure. To address the need for further information on the effects of isoflavones on cultured fish, the present study exposed first-feeding Rainbow Trout larvae to increasing levels of dietary genistein. The objectives of this study were to determine any effects dietary genistein may on growth and/or condition of fed Rainbow Trout fry and if the larval endocrine system would exhibit an estrogenic response as characterized by the VTG biomarker.

Methods and materials

Experimental diets and fish

Three experimental diets were formulated with additions of commercial SBM at 20 g/100 g diet and increasing levels of genistein at 0.0 (NoGen), 0.124 (LoGen), and 0.249 g/100g (HiGen); a reference diet (Ref) containing 20 g/100 g of fish meal with no SBM or genistein was also formulated for comparative purposes (Table 3-1). Each

experimental diet was balanced (dry matter basis) with a combination of whole cleaned wheat, whole yellow corn, and wheat gluten to produce similar proximate composition values of crude protein [42.2 ± 0.4% (mean ± SD)], crude lipid (17.3 ± 0.2%), crude fiber (3.03 ± 0.47%), and total ash (8.80 ± 0.49%) to meet the nutritional requirements of larval-juvenile Rainbow Trout (Table 3-1). The commercially produced SBM, HP 300, was obtained from Hamlet Protein Inc. (Findlay, Ohio). This SBM was analyzed for proximate composition by the South Dakota Agricultural Laboratories (SDAL, Brookings, South Dakota) and phytoestrogen levels of genistein, daidzein, equol, formononetin, and biochanin A by the United States Geological Survey (USGS), Organic Geochemistry Research Laboratory (OGRL, Lawrence, Kansas) prior to use in the experimental diets (Table 3-2). Pure genistein (>99%, $C_{15}H_{10}O_5$) was obtained from BioTang Incorporated (Lexington, Massachusetts). Whole yellow corn, tryptophan, calcium propionate, and betaine were provided by South Dakota State University, Department of Natural Resources Management (Brookings, South Dakota); additional information on these ingredients is listed in Table 3-1. All remaining ingredients were provided by Prairie Aquatech (Brookings, South Dakota) from their existing inventory and are listed with manufacturer information in Table 3-1.

Prior to extrusion, all dry ingredients, except genistein, were measured using calibrated scales and mixed for 5 min in a double action mixer (model L-200 DA, Leland Southwest, Fort Worth, Texas). Upon completion of mixing, each diet blend was ground with a Fitzpatrick Comminutor (model DAS06, Fitzpatrick Company, Elmhurst, Illinois) fitted with a 0.5-mm diameter, round-hole screen in order to grind large ingredient particles (i.e., corn and whole wheat) into smaller particles. Genistein was added

following this process to ensure no thermal degradation occurred by placing each experimental diet blend into a clean, plastic cement mixer (Kobalt, model SGY-CM1, Lowe's Companies, Inc., Mooresville, North Carolina), and adding two, one-half measurements of the estimated genistein concentration with 3 min of mixing between each addition. Following the addition of genistein, the three lipids used in the diet blends were measured into a common container and added to the dry diet blend in the plastic cement mixer in one-third rations with approximately 1-2 min of mixing occurring between lipid additions. Water was then added in one-half rations followed by approximately 1-2 min of mixing to produce an average total moisture content of 7.37 ± 0.51%.

To ensure no/limited thermal degradation of the added genistein, all diets were cold extruded using a Hobart 4146 grinder (Hobart Company, Troy, Ohio) with 2.38 mm die openings, where the only heat source occurred from mechanical shear (barrel temperature not taken). Each diet blend was added to a variable speed metering screw, which fed the blend at a constant rate (16.99 rpm) to the grinder. The grinder die end was fitted with a variable speed cutting head set at 35.3 rpm to produce pellets with approximately equal length to diameter measurements. Pellets produced from the grinder and cutter head were collected on dry pans, spread evenly across the pan, and placed on a drying rack. All diets were allowed to air dry overnight followed by light sieving to remove any fine particles from the pellets prior to placement in clean plastic bags for storage. All diets were initially placed in a -4°C refrigerator for 1 week then moved to a -20°C storage freezer until processed into smaller sizes or used to feed fish. Every step during mixing and extrusion was performed from low to high concentrations of genistein

to prevent or minimize overlap in genistein concentrations. Due to the use of larval fish and the extended growth period used in this study, pellets for each diet were ground into smaller particle sizes using a small food processor (Oster, model FPSTMC3321, Sunbeam Products Inc., Boca Raton, Florida) and sized using nested sieves and a laboratory test sieve vibrator (Derrick Screens, model 150, Derrick Corp., Buffalo, New York). Pellets were separated into the following sizes: 0.25-1.00 mm (PS1), 1.00-2.00 mm (PS2), and >2.00 mm (PS3). Samples of diets (PS1-sized) were analyzed for overall proximate composition (%, dry matter basis) by the SDAL, which included crude protein, crude lipid, crude fiber, total ash, nitrogen free extract, and total dry matter (Table 3-1). Similarly, each diet (PS1-sized) was analyzed by OGRL for the following phytoestrogens: genistein, daidzein, equol, formononetin, and biochanin A (Table 3-1). See Appendix A for a detailed description of the OGRL diet analysis.

Prior to initiation of this feeding trial, a pilot experiment (results not reported) was performed to evaluate fish culture and experimental protocols for use in this current study. Based on the pilot study, fertilized, eyed Rainbow Trout (Fish Lake strain) eggs were obtained from the United States Fish and Wildlife Service, Ennis National Fish Hatchery, Ennis, Montana, and hatched in a vertical-flow, tray incubator at a water temperature of 11.5 ± 0.1°C. At 17 days post-hatch (dph), larval Rainbow Trout were transferred to two, 210-L square polyethylene tanks and were acclimated to the experimental test water via daily water renewals and fed the PS1 Ref diet for a 4-day period. It is probable that most of the PS1 Ref diet remained uneaten as >50% of the fry (observation) did not swim-up until one day prior to or on 0 day of experiment (DOE) in either acclimation tank; this behavior in combination with the level of yolk absorption

were used as visual guides for initiation of exogenous feeding (Piper et al., 1982). Therefore, most larvae exposed to the treatment diets at the start of the feeding trial were likely first-time feeding larvae. After this acclimation period, 200 Rainbow Trout larvae (initial individual weight = 109 ± 22 mg and length = 25.7 ± 1.5 mm) were impartially and randomly stocked into each glass aquaria (n = 24) providing six replicate aquaria per diet. Acclimation practices and stocking dates were offset by 1 day between the two polyethylene acclimation tanks with the initial 12 aquaria (all treatments, 3 replicates each) stocked from one acclimation tank and the remaining 12 aquaria (all treatments, 3 replicates each) stocked from the staggered acclimation tank the following day. This stocking method provided staggered feeding, sampling, and collection periods for the duration of the study while providing similar DOE comparisons to be made for all replicates. Fish were fed the experimental diets to apparent satiation, based on standard feeding rates (Piper et al., 1982) and daily visual inspections for uneaten feed, three times daily for a 112-day feeding trial beginning at 23-24 dph. Feed amounts and pellet size fed was based on average individual weights and guidelines provided by the Food and Agricultural Organization of the United Nations (FAO, Rome, Italy) (FAO, 2020). The feeding trial initiation and duration coincided with the period of sexual differentiation which begins shortly after the yolk sac is completely absorbed and swim-up activity occurs (18-28 days at 11.5°C according to Billard 1992). Termination of the feeding trial occurred 112 days later and included the period when gonads are undifferentiated (until 48 dph), number of germ cells is increasing (until 70 dph), and ovary development is complete (until 84-112 dph) (Billard, 1992).

Length and weight data of individual fish and tank weights were measured at the initiation and 14-day intervals for the duration of the feeding trial (Table 3-3). In general, sampling for this data occurred as follows: 1) all fish were netted from an aquarium and placed in a plastic container filled with culture water from the aquarium, 2) a subsample (random and/or graded; Table 3-3) were removed and placed in a separate plastic container for individual length and weight measurements, 3) remaining tank fish were poured into a large mesh net suspended over another culture-water filled plastic container, 4) the bottom of the net was blotted with paper towel to remove access water and the fish transferred to an appropriate-sized, tared graduated cylinder, and 5) the fish were returned to their corresponding aquarium by inverting graduated cylinder and rinsing it with culture water. Returned fish were then visually observed for any potential negative effects that may have occurred from handling (i.e., loss of equilibrium, erratic or abnormal swimming behavior, etc.) and were replaced by subsampled fish of approximately the same size if such effects were seen. Subsampled fish from step two mentioned above were then processed as follows: 1) fish were euthanized, 2) total lengths (± 0.5 mm) were measured with a miniature measuring board, 3) fish were blotted with laboratory tissue paper to remove excess water and placed in a tared weighing dish to measure individual weights (± 1 mg), 4) all subsample fish were then transferred to an appropriate-sized, tared graduated cylinder, and 5) the graduate cylinder was then weighed to determine total subsample weight (± 0.01 g). The removal of subsamples at each sampling period was performed to provide treatment growth rates, keep stocking densities and water quality parameters within test aquaria at acceptable levels, and were adjusted to ensure enough fish remained for all necessary sampling periods and protocols

(Table 3-3). Also, the use of non-feed trained, first-feeding larval fish resulted in an apparent lack of feeding behavior, emaciation, slow-growth, and/or diseased larvae from 0-42 DOE. As a result, larvae exhibiting such characteristics were graded out during the subsampling collection for the 14, 28, and 42 DOE sample periods and were not used in metric calculations; numbers of sampled and graded larvae are listed in Table 3-3. Tank weights not including the subsampled fish were used to calculate new feeding rations. All fish euthanizations that occurred within this study were performed with a lethal dose (1,000 mg/L) of tricaine methanesulfonate (Tricaine-S, Western Chemical Incorporated, Ferndale, Washington). Due to the continued sequential removal of larvae/fish during the feeding trial (Table 3-3), individual fish weights were used to calculate replicate means (± SD) to estimate the following growth parameters: length and weight growth trajectories, estimated individual weight gain (IWG, g) = (final replicate mean weight – initial individual mean weight) and specific growth rate (SGR, %) = 100 × [(ln final replicate mean weight – ln initial individual mean weight) / rearing days] (Barnes et al., 2014). Individual fish length, weight, and liver weights were used to determine the following condition metrics: hepatosomatic index (HSI, %) = 100 × (liver weight / total fish weight) (Strange, 1996) and Fulton condition factor (K) = 10^5 × [total fish weight / (total fish length)^3] (Anderson & Neuman, 1996). Liver color scores were noted but not statistically analyzed as all but one liver (yellowish) were pinkish red in color. Mortality (%) was also calculated as the number of fish removed due to death, disease, and/or abnormal behavior divided from the number of initially stocked fish. Feed consumption was observed daily in all aquaria to ensure appropriate feeding rates and also to monitor fish health and water quality. Based on these observations, it appeared that fish in every

test aquarium consumed all the diet fed for the duration of feeding trial except from 47-53 DOE in one Ref diet fed aquarium when only about half of the fish appeared to actively feed, leaving uneaten feed. This reduced consumption was attributed to increased total ammonia levels that occurred during this time period. Corrective measures were taken (i.e., reduced feeding rations and subsample of fish removed to decrease biomass in all aquaria) and normal feeding activity with complete consumption of diet within this aquarium resumed at 53 DOE.

Along with above mentioned samples, blood samples were collected from all remaining fish 9 days following termination of the feeding trial to determine plasma vitellogenin (VTG) levels. Blood samples were collected from each fish via severing the caudal vein and collecting blood into heparinized, micro-hematocrit, capillary tubes (22-362-566, Fisher Scientific, Pittsburgh, Pennsylvania) after anesthetizing the fish with Tricaine-S. Capillary tubes were placed in a micro-hematocrit centrifuge (model MB, International Equipment Company, Needham Heights, Massachusetts) and spun at 13,000 g for approximately 2 min to separate plasma and red blood cells. Processed capillary tubes for each fish were placed together in a labelled cryovial (T310-5A, Simport, Bernard-Pilon Beloeil, Quebec, Canada) and stored at -80°C until analyzed. Plasma VTG levels were analyzed and determined using the Rainbow Trout enzyme-linked immunosorbent assay (ELISA) kit produced by Biosense Laboratories (V01004402, Bergen, Norway). Plasma was collected from micro-hematocrit tubes by breaking the tubes above the red-blood cell/plasma separation point and using a pipette (P20, Gilson Incorporated, Middleton, Wisconsin) with a clean, sterile tip to force plasma out of the capillary tube into a labelled, microcentrifuge tube (05-408-120, Fisher Scientific,

Pittsburgh, Pennsylvania). Plasma samples were then processed according to the standard operating procedures supplied with the ELISA kit and absorbances determined at 405 nm wavelength with a microplate reader (SpectraMax 190, Molecular Devices LLC, San Jose, California) and its corresponding software program. Plasma VTG concentrations were calculated from a standard curve obtained from absorbance values measured following the standard operating procedures of the ELISA kit. This study was performed in compliance with the South Dakota State University Institutional Animal Care and Use Committee (18-080A).

Culture System

The feeding trial was conducted in a flow-through proportional diluter system operated to deliver approximately 1 L (overall mean = 1.013 ± 0.005 L) of test water to each aquarium. Initial delivery rates were set for every 15 min, as per results from the pilot study, until 56 DOE when the delivery rates were increased to every 10 min due to increased ammonia levels in some test aquaria. Glass aquaria measuring 75 x 30 x 30.5 cm (68.6 L capacity) with 18-19 cm standpipes were used to maintain a mean volume of 45.9 ± 1.1 L; polyvinyl chloride (PVC) sleeves with slits cut at the bottom and wrapped in stainless steel mesh were placed over the standpipes to prevent larval impingement or loss from aquaria. All aquaria were set in two connected water-baths, which were filled with chilled reverse osmosis water to maintain similar culture temperatures (13.3 ± 0.3°C, range = 12.5-14°C) across all test aquaria. Culture temperatures within the water-baths were maintained by two liquid conditioning circulators (model CFF-330 and CFF-500, IMI Cornelius Remcor Incoporated, Glendale Heights, Illinois) and a water chiller (model 0.5HP, Penguin Chillers, Knoxville, Tennessee). Likewise, two submersible

utility pumps (model 2, Danner Manufacturing Incorporated, Islandia, New York) were used to circulate water between and through both water-baths to decrease water temperature differences between test aquaria. However, both pumps were removed at the 86-87 DOE to decrease any possibility of stray electrical voltage affecting test fish as abnormal conditions and swimming behavior were being observed randomly throughout test aquaria. Stray voltage was suggested as a possibility for these effects (Ron Hopper, Acting Project Leader, Ennis National Fish Hatchery, Ennis, Montana, personal communication). Test aquaria were randomly stratified in each water-bath so all treatments were represented in each water bath with equal number of replicates (i.e., each water-bath contained all 4 treatments with 3 replicates each). A photoperiod of 12:12 h light:dark was maintained using fluorescent (cool-white and wide spectrum) lighting controlled by a Smart Electrician® digital timer (model 368-1726, Menards Incorporated, Eau Claire, Wisconsin). A constant supply of oxygen was supplied using a regenerative blower (model WW80, Pentair Aquatic Eco-systems Incorporated, Apopka, Florida) and 10.2 cm air stones (model AM4, Penn-Plax, Hauppauge, New York).

To ensure adequate water quality was maintained throughout the duration of the feeding trial, both *in situ* and bench water quality parameters were measured. *In situ* measurements of conductivity (µS/cm), dissolved oxygen (DO; mg/L and % saturation), and temperature (°C) were taken with a portable multi-meter (model HQ40d18, Hach Company, Loveland, Colorado) fitted with conductivity (model CDC401-03, Hach Company, Loveland, Colorado) and DO (model LDO101-03, Hach Company, Loveland, Colorado) probes. Likewise, pH and temperature (°C) was measured *in situ* with a portable pH meter (model Orion™ Star™ A221, Thermo Fisher Scientific Incorporated,

Beverly, Massachusetts) fitted with a pH electrode (model 8107UWMMD, Thermo Fisher Scientific Incorporated, Clemsford, Massachusetts). *In situ* measurements were taken in all test aquaria held within 1 water-bath (i.e., 12 aquaria total) one day with the remaining aquaria being measured the following day; this alternating process was continued for the duration of the study. Daily mean culture water temperatures were recorded as the mean temperature measurements taken from all three portable meter probes and were used to calculate overall mean culture temperature. Discrete sub-surface grab samples were collected into 500- or 1,000-mL polyethylene bottles once per week from one replicate aquaria in each treatment. Samples were analyzed for alkalinity, hardness, and calcium using standard titrimetric methods from the American Public Health Association (APHA) (APHA, 1995); magnesium was calculated as the difference between total hardness and calcium. Additionally, another set of grab samples was collected into 130 mL polyethylene bottles, acidified in 0.4% reagent grade sulfuric acid (pH < 2), and placed in 4°C storage until analysis for total ammonia concentrations. Total ammonia as nitrogen (N) was measured in 50 mL samples using the same portable pH meter mentioned above fitted with an ion-selective electrode (model 9512HPBNWP, Thermo Fisher Scientific Incorporated, Clemsford, Massachusetts) following standard procedures recommended by the manufacturer and standardized APHA methods (APHA, 1995). The reporting limit for total ammonia was set at 0.1 mg/L as N corresponding to the lowest ammonia standard analyzed. Analysis of duplicate and spiked-reagent water samples were used as quality control measures to assess precision and accuracy, respectively, for both sets of collected grab samples. Table 3-4 lists all the calculated mean (± SD) and range of water quality parameters measured for this study.

To eliminate any potential chemical interference from culture water, deionized water was used to prepare synthetic reconstituted water types using prescribed/calculated amounts of food-grade or better salts. Synthetic reconstituted water types were prepared according to standards set forth by the United States Environmental Protection Agency (USEPA, 2002) (Table 3-5). Based on the previously mentioned pilot study, Rainbow Trout cultured in synthetic reconstituted soft water showed no negative effects or mortality rates; therefore, this water type was initially used to culture test fish until 56 DOE. However, due to observed negative effects and low-level mortality in test aquaria, reconstituted moderately hard water was used for the remainder of the feeding trial. Reconstituted waters were mixed and circulated in 11,356 L polyethylene tanks (Poly Processing Company Incorporated, Monroe, Louisiana) fitted with magnetic drive mixing pumps (model WMD-40RLT-115, Iwaki America, Holliston, Massachusetts) and provided aeration from an oil-less air compressor (model 7HDD-70TA-M750X, Gast Manufacturing Incorporated, Benton Harbor, Michigan) and air diffusers (model AS15L, Pentair Aquatic Eco-systems Incorporated, Apopka, Florida). Prior to use in the feeding trial, each blended tank of reconstituted water was thoroughly mixed, sampled, and analyzed for selected water quality parameters, including hardness, alkalinity, calcium, magnesium, and conductivity (Table 3-5). Sub-surface grab samples from blended tanks were analyzed using the same procedures as listed above for the aquaria culture water.

Statistical Analyses

All statistical analysis was performed using R (version 4.0.2) with an RStudio interface. The packages *agricolae, nortest, statmod, ggplot2, gridExtra,* and *grid* were used for statistical analysis and graphics design; all statistical analyses performed with α

= 0.05. Both length and weight growth curves were analyzed using *growthcurve* in the package *statmod*. All data used for remaining statistical analyses underwent an initial test for normality using the Shapiro-Wilk normality test. If the test indicated that the data was not normally distributed ($P < 0.05$), the metric was analyzed with a non-parametric Kruskal-Wallis (KW) test and a post-hoc pairwise-comparison (PWC) test if significant differences were detected between treatment means. Additionally, all metrics not normally distributed were *natural log* + 1 transformed and re-evaluated for normality in an attempt to use parametric statistical tests; however, transformed metrics remained not normally distributed after such transformations and resulted in the use of the non-parametric methods mentioned above. In contrast, normally distributed metrics were analyzed using a general linear model (GLM) and a post-hoc least significant difference (LSD) test if significant differences were detected between treatment means. All metrics were separated into two data sets and analyzed separately; one data set included all the treatments and the other removed Ref treatment data to analyze potential differences between the SBM-based diets only. The non-parametric statistical tests were used on all metrics, except mortality, when Ref data was included due to non-normal distribution, while conversely, all metrics were normally distributed with the removal of Ref data allowing for parametric statistical testing.

Results

Prior to any statistical analyses, an attempt was made to determine if plasma VTG levels sampled at 112 DOE could be used to identify sex of the cultured Rainbow Trout via frequency histograms. It was assumed that a bimodal distribution would be indicative of grouping males from females; however, only data from the Ref diet showed a weak

bimodal distribution with no apparent bimodal peaks in any of the SBM-derived diets (Figure 3-1). Therefore, no further attempts were made at analyzing sex determination and any corresponding differences.

Growth rates of mean individual fish total length and weight were plotted and showed a distinctively higher growth rate for fish fed the Ref diet starting at 14-28 DOE (Figure 3-2). Pairwise comparisons between the grouped growth curves via a permutation test indicated that both length and weight growth curves were statistically higher (P = 0.0132) for fish fed the Ref diet than all other diets. Similarly, both length and weight growth curves of fish fed the NoGen and HiGen diets were statistically higher and lower ($P < 0.0294$) than all other SBM-based diets, while those fed the LoGen diet were not statistically different ($P > 0.1018$) than NoGen and HiGen. This observation was verified by statistically higher IWG ($P = 0.013$) and SGR ($P = 0.013$) of fish fed the Ref diet, while fish fed the HiGen diet were statistically lower ($P < 0.026$) for both growth parameters when compared to those fed the Ref and NoGen (Table 3-6). Both parameters for fish fed the LoGen diet were not statistically different ($P > 0.130$) than the NoGen and HiGen diets. Likewise, when comparing only SBM-based diets, fish fed the HiGen diet had the statistically lowest ($P < 0.0401$) IWG and SGR (Table 3-6). Condition indices (i.e., HSI and K) showed no statistical difference ($P > 0.206$) between diets except for a statistically higher ($P = 0.013$) K value for fish fed the Ref diet (Table 3-2). Similarly, mortality rates were not significantly different ($P > 0.226$) between treatments.

Several of the samples (n = 15 of 326) had Vtg levels below detectable limits (i.e., <0.01) of the method used; all of these samples occurred in the NoGen treatment. These samples were given the value of 0.01 for all statistical analyses as this would be a

conservative value for such analyses. Results from the plasma VTG concentrations indicated several statistical differences ($P < 0.030$) with fish fed the HiGen and NoGen diets having the highest and lowest levels, respectively, while no statistical difference ($P = 0.937$) occurred between VTG levels for fish fed the Ref and LoGen (Figure 3-3).

Discussion

Results from this study provided evidence for the negative effects that dietary SBM inclusion and increasing levels of dietary genistein can have on larval-juvenile Rainbow Trout. Regarding growth, IWG and SGR rates showed that Rainbow Trout fed the Ref diet clearly outperformed those fed any SBM-based diet. Likewise, condition indices showed identical results with Ref diet fed fish maintaining statistically higher condition values. Since all diets maintained similar fish meal levels, the greatest dietary differences occurred with the replacement of wheat gluten in the Ref diet with SBM in the treatment diets. This difference in dietary component inclusion most likely resulted in reduced growth and condition as a result of SBM-induced enteritis (e.g., inflammation of the distal intestine). These negative outcomes have been studied and reported in a variety of studies on Rainbow Trout and other salmonid species with the inclusion of soy-based proteins (Bakke-McKellep et al., 2007; Booman et al., 2018; Bureau et al., 1998; Kaushik et al., 1995; Krogdahl et al., 2015; Olli & Krogdahi, 1994; Van den Ingh et al., 1991, 1996). In contrast, studies have shown no observed antinutritional effects with the inclusion of wheat gluten as one of the primary protein sources in the diets of Rainbow Trout (Apper-Bossard et al., 2013; Storebakken et al., 2000) or Atlantic Salmon (Apper-Bossard et al., 2013; Rodehutscord et al., 1995). Therefore, it is apparent that Rainbow Trout in this study exhibited greater negative impacts on digestive functions

and/morphology due to the ANFs found within the used SBM; however, no attempt was made to determine type and level of differing ANFs beyond several phytoestrogens.

Along with the dietary differences, the limited use of extrusion processes to make the experimental diets may have also played a role in the decreased growth performance and condition of fish fed the SBM-based diets. As mentioned in the methods section, the only heat source during the extrusion process occurred from the mechanical sheer of the grinder to reduce any potential loss of genistein from thermal degradation. However, such processing may have resulted in higher levels of lectins and proteinase inhibitors as decreased levels of these ANFs occur following heat exposure from moisture, pressure, temperature, and mechanical shear during the extrusion process (Barrows et al., 2007; Gatlin III et al., 2007; Krogdahl et al., 2010). Such increased levels of these ANFs would have led to the same deleterious effects of reduced protein digestibility from the affected digestive functions and morphology mentioned above. Based on these observations and the current data, it is highly advised that aquaculture diets used for young Rainbow Trout undergo adequate extrusion processing to reduce associated ANFs and thereby, increase growth potential and fish condition. Likewise, increased temperatures may also result in lower concentrations of genistein as thermal degradation has been noted at high temperatures (120°C) (Ungar et al., 2003), and thus, would result in reduced negative effects from dietary genistein, which will be discussed next. However, care should be taken with extrusion processing as overheating may damage SBM proteins and decrease protein digestibility and amino acid availability (Barrows et al., 2007).

When comparing results from the SBM-based diets only, increased genistein levels did have a negative impact on the growth of reared Rainbow Trout with the HiGen

diet having significantly lower IWG and SGR. Conversely, increasing genistein levels did not appear to affect the overall condition of the reared Rainbow Trout as no differences were seen. Several studies showed similar growth and condition results of fish fed the NoGen and LoGen diets; however, genistein inclusion levels were below those used in this study. Juvenile Rainbow Trout (initial weight = 40 g) showed no decreased growth performance when fed diets containing 0-1,000 mg genistein/kg diet (Bennetau-Pelissero et al., 2001). Adult Japanese Medaka fed increasing levels of genistein from <0.8-58.5 mg/kg diet exhibited no differences in total length, body weight, or HSI (Inudo et al., 2004). Juvenile Goldfish (initial weight = 6.4-13.4 g) experienced no significant differences in initial/final body weight or HSI when fed diets containing up to 390.8 mg genistein/kg diet (Ishibashi et al., 2002). Several studies examined growth and/or condition of fish fed diets containing genistein at levels greater than the current study with varying results. Juvenile Rainbow Trout fed 3,000 mg genistein/kg diet maintained similar final body weights, SGR, and HSI values to fish fed a control diet; however, the authors noted that overall growth of fish within the study was poor with only a 41-69% weight increase (Torno et al., 2019). Female Yellow Perch (initial weight = 13-15.5 g) had significantly lower weight and length measurements compared to controls when fed 7,500 mg genistein/kg diet, while the growth of males was not statistically different when fed either 750 or 7,500 mg genistein/kg diets (Ko et al., 1999). Channel Catfish (*Ictalurus punctatus*) fry fed 0, 2,000, 4,000, and 8,000 mg genistein/kg diet showed no differences in HSI between treatments; growth was not analyzed (Green & Kelly, 2009). Another study, with two feeding trials, using the same inclusion levels found no significant differences in HSI of juvenile Striped Bass in either

trial; however, significantly lower body weight gains were seen in fish fed the 4,000 mg/kg diet in one trial, while the other trial had an observational decrease in body weight gains at the same treatment level (Pollack et al., 2003). The growth results of SBM-based diets in this study, along with comparative studies mentioned above, seemed to indicate that dietary levels of genistein above 1,500 mg/kg do result in some degree of decreased growth performance, while having no apparent impact on condition as denoted by HSI. Also, Ko et al. (1999) found that utilizing appropriate levels of dietary E2 improved growth of Yellow Perch over controls and suggested that genistein may have the similar capacity to enhance growth. However, the authors indicated that this outcome would be dependent on determining the appropriate dietary concentration and that too high of concentrations would lead to reduced growth rates as seen in their results (Ko et al., 1999) and in part, may explain the reduced growth observed in the current study.

Additionally, several possible explanations for this reduction in growth performance from the HiGen treatment exist based on studies performed on Atlantic Salmon and Nile Tilapia. The first study analyzed the growth performance, digestive enzyme activities, and intestinal and liver morphology of Atlantic Salmon fry from first-feeding to juvenile growth stages when fed diets containing soybean various ANFs (Gu et al., 2015). One diet contained 1,500 mg isoflavones/kg diet; however, types and levels of isoflavones within the diet were not reported (Gu et al., 2015). When compared to the control and other treatment diets containing isoflavones, soya-saponins (SAP), phytosterol (PHS), or a mixture of all 3 ANFs, the fish fed the isoflavone diet had the lowest observed final body weight (i.e., statistically lower than the SBM-control, SAP, and PHS diets), significantly lower maltase and higher trypsin activities in the proximal

intestine compared to the FM-control and SAP diets, and significantly lower hepatocyte vacuolization and diameter size (Gu et al., 2015). The authors concluded that the isoflavone diet induced an alteration of the digestive enzymes and suggested an inhibitory capacity on thyroid synthesis as the reason for the reduced growth performance (Gu et al., 2015). Likewise, the authors suggested that depleted glycogen stores in hepatocytes from the isoflavone diet may have been an outcome of the reduced digestive function or reduced amount of blood glucose available to the body for fuel resulting in decreased growth (Gu et al., 2015). In the other study, juvenile Nile Tilapia (initial weight = 10.47 ± 1.24 g) were fed 0, 30, 300, and 3,000 mg genistein/kg diet to evaluate growth performance and digestive enzymes activities (Chen et al., 2015). Growth results indicated fish fed the highest genistein inclusion diet had significantly lower final body weights and SGRs compared to the control fish (Chen et al., 2015). In an effort to determine this reduced performance, the authors analyzed the digestive enzyme activity of the experimental fish and, when compared to control fish, found decreased enzyme activities in the following: 1) protease in the stomach (300 and 3,000 mg/kg treatments) and hepatopancreas (3,000 mg/kg treatment), and 2) amylase in the liver (3,000 mg/kg treatment) and intestine (300 and 3,000 mg/kg treatments) (Chen et al., 2015). The authors suggested these results depicted decreased growth due to the inhibitory effect of genistein on digestive enzymes activities and warned of the use of soybean products as alternative ingredient sources in aquaculture diets. While no attempt was made to determine the potential gut morphology and/or the digestive microbial community within this study, it is probable that SBM-induced enteritis mentioned above coupled with the inhibitory effect of genistein on digestive enzymes activities resulted in reduced growth

performance of the larval Rainbow Trout in this study. Likewise, it is highly probable that the larval to juvenile life stages used in this feeding trial may have contributed to a greater effect on growth as compared to most of the above studies utilizing juvenile to adult stage fish. This effect is most likely due to the digestive ontogeny from larval to juvenile stages seen in the dynamic and complex processes of organ morphogenesis, functional development of digestive, and adsorptive functions that have been observed and described in other studies (Rønnestad et al., 2013; Sala et al., 2005).

Mortality rates indicated genistein did not affect survival of larval-juvenile Rainbow Trout as no differences occurred in any of the treatments. Similar results have been reported in other studies analyzing dietary genistein fed to Nile Tilapia (Chen et al., 2015) and Guppies (*Poecilia reticulata*) (Chakraborty et al., 2012) and waterborne exposure of genistein to male Siamese Fighting Fish (*Betta splendens*) (Stevenson et al., 2011) and Fathead Minnows (*Pimephales promelas*) (Ingham et al., 2004). In contrast, two other studies did report decreasing survival in Nile Tilapia fed genistein at 20 and 30 mg/kg diet (combined with 60 µg 17α-methyltestosterone/g in each diet) (El-Sayed et al., 2012) and poor survival (28%) of Southern Flounders (*Paralichthys lethostigma*) fed 1,000 mg genistein/kg diet (DiMaggio et al., 2016). This contradiction again points to the varying effects of genistein on differing species and life-stages, which merits caution when genistein is used within diets and warrants further research. However, at present, it appears that the presence of genistein within Rainbow Trout diets for any life-stage does not have deleterious effects upon survival.

As mentioned in the results section, histograms were utilized to try and distinguish male and female specimens within each treatment. While this approach did

produce some bimodal evidence of sexual differentiation within the Ref diet, such evidence was minimal or non-existent in SBM-diet treatments. This fact is most likely due to the use of sexually immature fish as Pollack et al. (2003) stated that VTG production can be indicative of exposure to estrogenic substances in both immature females and males as only mature females normally produce VTG for oocyte development. However, these results provide evidence that the physiological system of larval-juvenile Rainbow Trout have the capacity of recognizing genistein as an estrogenic substance and reacting to it via increased or decreased VTG production.

Results of the VTG response to genistein provided very distinguishable differences between the treatment groups. The most poignant difference occurred with fish fed the HiGen diet exhibiting significantly higher levels of plasma VTG with levels 3 to 6 times greater than fish in the other treatments. Similar increases in VTG production have been noted in several different studies with various fish species. Male Rainbow Trout (initial weight = 40 g) exhibited significantly higher plasma VTG levels at 2 to 3 times greater than the control for an entire year, except during the spawning period, when fed 500-1,000 mg genistein/kg diet (Bennetau-Pelissero et al., 2001). Adult, male Japanese Medaka had significantly higher hepatic VTG production when fed a commercial diet containing 58.5 mg genistein/kg and 37.3 mg daidzein/kg (Inudo et al., 2004). Male Goldfish (initial weight = 6.4-13.4 g) fed an ornamental carp diet containing 390.8 mg genistein/kg and 416.8 mg daidzein/kg produced significantly higher plasma VTG levels than those on a control diet containing 93.2 ng genistein/g and 129.6 ng daidzein/g (Ishibashi et al., 2002). Striped Bass fingerlings produced significantly higher VTG levels when fed diets containing 2,000 and 8,000 mg genistein/kg compared to the

control and 4,000 mg genistein/kg (Pollack et al., 2003). In contrast, several studies showed no difference in Rainbow Trout VTG production with inclusion of dietary genistein; however, most used genistein levels well below those of the HiGen diet and the 500-1,000 mg/kg diets used in Bennetau-Pelissero et al. (2001). Juvenile Rainbow Trout (initial weight = 83 ± 1 g) fed diets containing 2.9-105.0 mg genistein/kg and 5.4-61.0 mg daidzein/kg diet showed no differences in circulating VTG levels (Kaushik et al., 1995). In another study, Rainbow Trout (mean weight = 350 g) fed 0.2-20 mg genistein/kg diet did not have any differences in VTG production when fed for an 8-week period (Latonnelle, Le Menn, et al., 2002). Similar to the growth results, the effects of genistein on VTG production appears to be correlated with species and higher levels of dietary genistein with >500 mg/kg necessary to induce VTG production in Rainbow Trout.

The VTG levels produced by fish fed the SBM-based diets provided a clear and statistical dose-dependent increase. Such an increase could be explained by the ability of genistein to act as an estrogen agonist through binding with estrogen receptors in an increasing manner due to increased available genistein. Alternatively, the effects of genistein on E2 metabolism within the liver and kidney may have produced increased VTG by increasing the bioavailability of E2 via inhibition of E2-metabolizing enzymes in these tissues. This potential mechanism was suggested in a study by Ng et al. (2006) where *in vitro* cultures of Rainbow Trout liver and kidney tissues exposed to 0.1-10 µM genistein produced a dose-dependent decrease in water soluble (i.e., conjugated) E2 metabolites. Another possible explanation for this increase could be from increased up-regulation of the vitellogenin gene (*vtg*) within liver tissue. A recent study on juvenile

Russian Sturgeons (*Acipenser gueldenstaedtii,* 100 dph) evaluated the effects of genistein (500 mg/kg) and daidzein (10,000 mg/kg) on the expression of various sex-related genes in various tissues including *vtg* in livers (Fajkowska et al., 2021). Results showed a distinctive up-regulation of *vtg* from the daidzein treatment facilitated by increased transcription of *erα* and *erβ* genes, while some up-regulation occurred in the genistein treatment (Fajkowska et al., 2021). These findings coupled with the increased VTG levels seen in this study lend further evidence to the agonist role of genistein and/or its ability to up-regulate sex-related genes in a dose-dependent matter. However, the overall ability and extent of phytoestrogens on gene expression and molecular pathways has yet to be clearly addressed as evidenced by minimal research and discussion within the literature.

While a dose-dependent relationship occurred for plasma VTG in the SBM-based diets, an interesting and paradoxical shift in plasma VTG became apparent when those diets were compared to the Ref diet. A unique U-shaped response curve occurred when VTG data from the Ref diet was included with fish fed the NoGen and HiGen diets having the statistically lowest (2 times less) and highest amounts of plasma VTG, respectively, than the Ref and LoGen diets, which were not statistically different from one another. This type of U-shaped response curve has occurred in other studies, albeit in slightly different patterns, and lends itself to support the dualistic role of genistein as an estrogen agonist and antagonist. As mentioned above, juvenile Striped Bass exhibited decreased VTG, similar to levels of control fish, when fed 4,000 mg genistein/kg diet (Pollack et al., 2003). The authors attributed this decrease to either an unknown mechanism, a low-dose effect related to EDC's as seen in laboratory mice (vom Saal et al., 1997), and/or the dual function of genistein as an estrogen agonist/antagonist (Pollack

et al., 2003). Similarly, this type of paradoxical effect occurred in adult Rainbow Trout where decreased levels of sex hormones (i.e., follicle stimulating hormone, luteinizing hormone, and 17α,20β$(OH)_2$-progesterone) occurred at the end of gametogenesis and during the spawning period when fed 500 mg genistein/kg compared to 0 and 1,000 mg/kg (Bennetau-Pelissero et al., 2001). Likewise, differences in VTG production between fish fed genistein versus control diets occurred throughout the sampling periods for both males and females (Bennetau-Pelissero et al., 2001). The authors indicated that the effect of genistein upon these results should be interpreted by its dualistic nature with agonist or agonistic effects determined by the ratio of endogenous E2 to genistein at the target organ or tissue (Bennetau-Pelissero et al., 2001). While VTG was not analyzed, another study reported a paradoxical sex ratio shift in larval-juvenile Channel Catfish (*Ictalurus punctatus*) where greater proportions of phenotypically male fish were seen along with intersex individuals when fed 4,000 and 8,000 mg genistein/kg diet compared to 0 or 2,000 mg/kg treatments (Green & Kelly, 2009). The authors of this study indicated that the paradoxical sex ratios may be due to the weak estrogenic properties of genistein, blocking E2 from receptor sites, and/or the inhibition of aromatase, which resulted in decreased E2 synthesis (Green & Kelly, 2009).

It is apparent from these results and the above-mentioned discussion that the role of genistein is not only that of an estrogen agonist, due to its similar chemical structure to E2, but also a possible partial agonist and/or antagonist of estrogen (Kurzer & Xu, 1997; Setchell & Cassidy, 1999). This dualistic role of genistein and its corresponding biological actions can be dependent on numerous factors such as estrogen receptor numbers and type (α vs. β) (Setchell & Cassidy, 1999), competing endogenous E2

concentrations at the target tissue/organ (Bennetau-Pelissero et al., 2001; Setchell & Cassidy, 1999), regulation of E2 metabolism (Ng et al., 2006), aromatase enzyme effects on E2 synthesis (Green & Kelly, 2009), and reduced potency and estrogen response compared to E2 (Green & Kelly, 2009; Latonnelle, Fostier, et al., 2002). While the purpose of this study was not to determine the specific pathways of genistein effects on the endocrine system, it does lend support to the continued discussion of its dualistic nature and can be explained within the current framework of its known biological actions. Several different possibilities exist for this phenomenon and are described here. For fish fed the NoGen diet, it is possible that genistein reduced the amount of endogenous E2 for binding, while simultaneously blocking and/or binding with available estrogen receptor sites. Such blocking/binding could have exerted minimal estrogenic effects (i.e., reduced VTG production) due to the weak estrogenic properties of genistein and its limited availability within the NoGen diet. Examples of the weak estrogenic nature of genistein have been described for such paradoxical effects on Channel Catfish where greater numbers of phenotypic males were seen (Green & Kelly, 2009) and Striped Bass that depicted a U-shaped VTG response curve due to a possible low-dose effect (Pollack et al., 2003); both studies are mentioned in more detail above. Similarly, genistein may have reduced the amount of available endogenous E2 via aromatase pathways and/or increased E2 metabolism resulting in decreased E2 production and consequently lower VTG levels. Several studies have reported an inhibitory effect on aromatase enzymes in fish when exposed to phytoestrogens, which is the enzyme responsible for converting androgens into estrogen compounds (Cheshenko et al., 2008). In particular, genistein was found to exert weak inhibitory effects on Rainbow Trout ovarian aromatase activity via an *in vitro*

study (Pelissero et al., 1996); therefore, genistein may have produced a greater *in vivo* inhibitory effect upon the larval-juvenile Rainbow Trout undergoing gonad ontogeny as compared to adult fish. It stands to reason that either one or a combination of these factors may have occurred within the studied Rainbow Trout. As such, further study of E2 synthesis and metabolism of different species and life stages is needed to gain a better understanding of the complex and dualistic role of genistein.

While the reduced VTG production in fish fed the NoGen diet has several possible explanations, the overall paradoxical response of VTG production to increasing levels dietary genistein is best interpreted by recognizing its role as an *in vivo* agonist and antagonist with the estrogenic effects determined by the ratio of endogenous E2 to genistein available at the target organ as suggested in other studies (Adlercreutz, 1990; Bennetau-Pelissero et al., 2001; Herman et al., 1995; Verdeal et al., 1980). Several possibilities exist for how the levels of endogenous E2 may have been affected by the level of genistein present. First, the low-dose and/or antagonist mechanism of genistein, mentioned above, occurring in the NoGen diet may have resulted as genistein levels increased, thereby increasing production of endogenous E2 and consequently VTG production levels. Second, it is possible that genistein continued to inhibit E2 production as seen in the NoGen treatment; however, the increased genistein levels available in the LoGen and HiGen may have overcome the weak estrogenic nature of genistein by activating estrogen receptors at levels similar to or greater than the Ref diet. Third, genistein exposure may have altered the metabolism of E2 within the peripheral tissues (i.e., liver and kidney) of the treatment fish. As mentioned earlier, Ng et al. (2006) described a mechanism where the estrogenic effects of genistein occurred from increased

bioavailability of E2 by inhibiting E2-metabolizing hormones within liver and kidney tissues (Ng et al., 2006). Therefore, it is possible this mechanism increased the availability of endogenous E2, whether it was inhibited or not by the antagonistic effects of genistein. Whether it was one or a combination of these suggested explanations, enough estrogen receptors were activated by available E2 and/or genistein for fish to produce VTG levels similar to or greater than that of the fish fed the Ref diet. This fact provides evidence for the possibility of using phytoestrogens as a balancing mechanism for endocrine functions when fish are fed SBM-based diets. Such a possibility would be beneficial to researchers and fish culturalists looking to reduce food costs with plant-based proteins without alteration of the endocrine/reproduction functions of the fed fish species. However, the need to determine appropriate inclusion levels and life stages for such use is necessary as this study and others depicted negative growth and physiological impacts when fed diets with genistein levels above or below species tolerance thresholds.

Conclusion

The research area of dietary isoflavone exposure and its effects upon teleost fish is one that continues to provide a variety of conflicting and paradoxical results as evidenced in the literature and this current study. Growth results from this current study supported previous evidence for the negative effects of dietary SBM inclusion and, to our knowledge, provided the first evidence of decreased growth from dietary genistein on first-feeding, larval Rainbow Trout through the juvenile life-stage. For both growth parameters, evidence from the literature would suggest that decreased digestive functions due to altered gut morphology and/or enzymatic activity are the probable causes. Condition factors appeared closely tied to growth and the inclusion of SBM, while HSI

values indicated no difference with SBM or genistein inclusion. While some conflicting data exists for growth and condition, it is apparent that increasing levels of dietary genistein above 1,500 mg/kg results in some negative growth performance and/or condition within Rainbow Trout and several other studied species. Dietary genistein does not appear to pose a significant risk to survivability for differing life stages of cultured Rainbow Trout; however, other species showed conflicting results with increased mortality from genistein inclusion. These differences point to the varying effects genistein can have based on physiological differences between species and the sensitivity of differing life-stages within a species. Also, some of this complexity of growth and condition within the literature can be attributed to the use of differing methodologies, particularly experimental diet formulations and genistein/isoflavone inclusion rates. Therefore, it is necessary for continued research on the effects of dietary isoflavones in order gain a better understanding of the physiological effects on growth performance, condition, and survival of differing species and life stages. However, it is suggested that the use of more standardized methods occur to facilitate easier comparisons and analyses of those effects. To that end, a compiled data set of species sensitivities and effects to genistein at its various inclusion levels should be determined and reported. From this initial dataset, sub-groups of species based on isoflavone sensitivity could be suggested; thereby allowing future research to use some set of standardized dietary inclusion levels of genistein based on each sensitivity sub-group. For example, the estimated threshold of 1,500 mg/kg mentioned above could be used as relative midpoint for dietary inclusion with rates occurring above and below on an exponential scale (i.e., 0, 375, 750, 1,500, 3,000, and 6,000 mg/kg) for the initial study of a new species or life stage and then

modified for later studies based on the observed sensitivity and effects of the species to genistein along with comparisons to species in a similar sensitivity sub-group.

Results from this study provide further evidence of the dualistic role of genistein in terms of estrogenic effects (i.e., plasma VTG production). When comparing only the SBM-based diets, a significant dose-dependent relationship occurred with increasing VTG production from increasing genistein inclusion, which suggested an agonistic effect. However, when compared with VTG levels from the Ref treatment, it is apparent that the NoGen diet experienced some type of antagonistic effect(s) resulting in significantly lower VTG levels. This antagonistic effect was replaced by an increasing agonistic effect in the LoGen and HiGen diets. The dualistic nature of genistein and its corresponding impacts on the physiological processes of teleost fishes appears to be a highly complex issue depended on a variety of factors from regulation of gene transcriptions, modifications to E2 metabolism, to direct competition for estrogen receptor sites, as previously mentioned. However, the results from this study, along with several others in the literature, seems to best explain this dualistic nature by the ratio of endogenous E2 to available genistein, where any of the discussed factors may have led to varying levels of endogenous E2. These levels in combination with the available genistein from diets resulted in decreased, normal, or increased VTG production of fish fed the SBM-based diets when compared to those in the Ref diet. Like the suggestions above for growth, continued research is warranted to study and determine the physiological mechanisms of genistein on different fish species, life-stages, and biological pathways. To this end, some additional study standardization would be highly beneficial to better illicit potential and/or overarching effects between and within species. For physiological studies,

genistein inclusion rates would be more difficult to standardize due to the wide range of conflicting effects seen within the literature. However, it is suggested that such studies analyze endogenous E2 levels and corresponding VTG production in order to determine the potential effects from the ratio of E2 to genistein. Along with this, it is highly likely that overt and/or delayed effects upon sexual development and/or reproductive fitness in growing Rainbow Trout may occur later in development as the current study provided statistical evidence to estrogenic effects from genistein exposure. Therefore, care should be taken to ensure limited amounts of genistein exist in aquaculture diets, particularly for larval fish during the period of gonadal development with consideration to overall culturing goals of fish (i.e., growth, broodstock, etc.).

Table 3-1. Composition (g/100 g, dry matter basis) of experimental diets (NoGen, LoGen, and HiGen) containing a commercial soybean meal and increasing levels of genistein and a reference (Ref) diet.

	Diets			
	Ref	**NoGen**	**LoGen**	**HiGen**
Ingredients				
Menhaden fish meal[a]	20.0	20.0	20.0	20.0
Soybean meal[b]	0.00	20.0	20.0	20.0
Genistein[c]	0.00	0.00	0.124	0.249
Pork blood meal[d]	3.00	3.00	3.00	3.00
Poultry byproduct meal[e]	15.0	15.0	15.0	15.0
Feather meal[f]	2.00	2.00	2.00	2.00
Whole cleaned wheat[g]	19.055	13.475	12.98	12.98
Whole yellow corn[h]	5.23	5.00	5.376	5.251
Wheat gluten[i]	15.93	2.68	2.68	2.68
CMC[j]	0.80	0.30	0.30	0.30
Vitamin mix[k]	1.70	1.70	1.70	1.70
Mineral mix[k]	1.50	1.50	1.50	1.50
Supplements				
Stay-C[l]	0.30	0.30	0.30	0.30
Choline chloride[m]	0.30	0.30	0.30	0.30
Lysine[n]	0.75	0.50	0.50	0.50
Tryptophan[o]	0.10	0.10	0.10	0.10
Methionine[p]	0.50	0.50	0.50	0.50
Betaine[q]	0.29	0.29	0.29	0.29
Taurine[r]	0.50	0.50	0.50	0.50
Calcium propionate[s]	0.025	0.025	0.025	0.025
Lipids				
Menhaden fish oil[t]	10.0	10.0	10.0	10.0
Soybean oil[u]	2.02	1.83	1.83	1.83
Corn oil[v]	1.00	1.00	1.00	1.00
Proximate Composition (%)[w]				
Crude protein	42.3	41.9	42.0	42.7
Crude lipid	18.4	17.1	17.4	17.5
Crude fiber	1.98	2.52	3.43	3.14
Total ash	8.19	8.25	8.97	9.18
Nitrogen free extract	20.7	23.4	20.4	20.0
Total dry matter	91.6	93.2	92.2	92.5
Phytoestrogen Concentration (µg/g)[w]				
Genistein	3.45	30.6	873	2,010
Daidzein	3.02	121	80.5	88.6
Equol	1.10	1.26	1.07	1.06
Formononetin	0.0012	0.0323	0.0372	0.0325
Biochanin A	<0.001	<0.001	0.0181	0.0274

[a] Lot 3202270, Omega Protein, Reedville, Virginia.

[b] HP 300, Hamlet Protein Incorporated, Findlay, Ohio.
[c] Lot 092118, Genistein >99%, Biotang Incorporated, Lexington, Massachusetts.
[d] Lot 19016, Consumer's Supply Distributing LLC, North Sioux City, South Dakota.
[e] Lot WK8, River Valley Ingredients, Hanceville, Alabama.
[f] Lot PO106, Tyson Foods Incorporated, Springdale, Arkansas.
[g] Locally sourced, Prairie Ag Partners, Brookings, South Dakota.
[h] Locally sourced, AgFirst Farmers Cooperative, Brookings, South Dakota.
[i] Lot 17280314, Cargill, Minneapolis, Minnesota.
[j] Carboxymethyl cellulose, Akzo Nobel N.V., Amsterdam, Netherlands.
[k] Proprietary blend from Prairie Aquatech, Brookings, South Dakota.
[l] Lot UEO1803044, DSM Nutritional Products Incorporated, Parsippany, New Jersey.
[m] Lot 60-799, BalChem Corporation, New Hampton, New York.
[n] Lot HAAF190217, CJ Bio America Incorporated, Fort Dodge, Iowa.
[o] L-Tryptophan, ChemSol LLC, Minnetonka, Minnesota.
[p] Lot 38J316, Sumitomo Chemical, Chuo City, Tokyo, Japan.
[q] Betaine hydrochloride, Pure Bulk, Rosenburg, Oregon.
[r] Lot C002YB1801496, Hubei Grand Life Science and Technology Company Ltd., Huangshi City, Hubei Province, China.
[s] 98+%, Acros Organics, Morris Plains, New Jersey.
[t] Lot E80315, Daybrook Fisheries Incorporated, Empire, Louisiana.
[u] Lot S180346V, South Dakota Soybean Processors LLC, Volga, South Dakota.
[v] Mazol, ACH Food Companies Incorporated, Oakbrook Terrace, Illinois.
[w] Analysis conducted on post-manufactured pellets.

Table 3-2. Composition (%, dry matter basis) and phytoestrogen concentrations (µg/g) of the commercially produced soybean meal protein HP 300 from Hamlet Protein Incorporated, Findlay, Ohio, used in this study.

Property/Phytoestrogen	**Values**
Proximate Composition (%)	
Crude protein	55.6
Crude lipid	4.16
Crude fiber	6.75
Total ash	5.04
Nitrogen free extract	25.1
Total dry matter	96.6
Concentration (µg/g)	
Genistein	230
Daidzein	591
Equol	0.0219
Formononentin	0.120
Biochanin A	0.0102

Table 3-3. Number (*n*) of planned, total sampled, and graded Rainbow Trout larvae/juveniles per replicate aquarium that occurred at the listed day of experiment (DOE) for this study. Comments are included indicating reasons for grading, differences in planned and sampled *n*, and data collected beyond standard individual length and weight data. Discrepancies between planned and sampled *n* occurred due to mortalities and in one case (16 fish at 98 DOE) an apparent miscount from the previous sampling period resulting in one extra fish within the replicate aquarium.

DOE	Planned *n*	Sampled *n*	Graded *n*	Comments
0	40	40	NA[a]	total number of larvae sampled, not per aquarium.
14	20	9-19	0-13	Removed larvae (Graded *n*) that were emaciated or diseased, per aquarium.
28	20	18-20	8-10	Removed larvae (Graded *n*) that showed slow growth.
42	20	17-20	4-6	Removed larvae (Graded *n*) that showed slow growth.
50-51	15	15	15	Removed larvae to decrease ammonia levels; graded as larger pellet sized used.
56	20	18-20	NA	Feeding trial mid-point. Pooled liver samples collected from 9 fish/aquarium.
63	10	9-10	NA	Pooled fecal samples collected from 8 fish/aquarium.
70	20	18-20	NA	
84	20	17-20	NA	
98	15	13-16	NA	Planned *n* reduced to 15 so enough remained for final sampling.
112	20	18	NA	End of feeding trial duration. Pooled liver samples collected from 9 fish/aquarium.
119	9	8-9	NA	Pooled fecal samples collected from 8 fish/aquarium.
121	NA	12-14	NA	Blood and tissue samples collected from all remaining fish.

[a] NA = not applicable.

Table 3-4. Bench and *in situ* water quality parameters of test aquaria culture water used in this study. Parameters listed under either reconstituted soft water (ReSoft) used from 0 to 58th day of experiment (DOE) or reconstituted moderately hard water (ReModHard) used for the remainder of the study. Mean ± standard deviation listed with the range shown inside parenthesis.

Parameters	**ReSoft (0-58 DOE)**	**ReModHard (59-121 DOE)**
Alkalinity (mg/L $CaCO_3$)	33.1 ± 1.3 (30.5-36.0)	62.4 ± 1.0 (61.0-65.0)
Hardness (mg/L $CaCO_3$)	42.4 ± 0.4 (42.0-43.0)	84.7 ± 0.6 (83.5-85.5)
Calcium (mg/L)	7.9 ± 0.2 (7.6-8.4)	14.7 ± 0.1 (14.4-15.0)
Magnesium (mg/L)	5.5 ± 0.1 (5.2-5.8)	11.6 ± 0.1 (11.3-11.9)
Total ammonia (mg/L as N[a])	0.486 ± 0.309 (0.019-1.285)	0.752 ± 0.234 (0.481-1.391)
Dissolved oxygen (mg/L)	9.51 ± 0.29 (8.31-10.13)	9.28 ± 0.34 (7.50-9.75)
Dissolved oxygen (% sat.[b])	94.7 ± 2.82 (82.7-99.8)	92.6 ± 3.3 (75.5-97.0)
Conductivity (µS/cm)	159.9 ± 3.1 (154.8-175.1)	303 ± 3 (296-311)
pH	7.63 ± 0.07 (7.46-7.85)	7.79 ± 0.08 (7.47-7.94)

[a] N = nitrogen.
[b] % sat. = percent saturation.

Table 3-5. Acceptable range (Range) and measured water quality parameters of synthetic reconstituted soft water (ReSoft) or reconstituted moderately hard water (ReModHard) used to culture Rainbow Trout in this study. Mean ± standard deviation listed with the range shown inside parenthesis.

	ReSoft		ReModHard	
Parameters	**Range[a]**	**Measured**	**Range[a]**	**Measured**
Alkalinity (mg/L $CaCO_3$)	30-35	31.2 ± 0.3 (31.0-32.0)	57-64	58.9 ± 0.8 (57.0-60.0)
Hardness (mg/L $CaCO_3$)	40-48	41.9 ± 0.4 (41.0-45.0)	80-100	84.5 ± 0.8 (82.5-85.5)
Calcium (mg/L)	NA[b]	7.6 ± 0.3 (7.0-8.0)	NA	14.6 ± 0.3 (14.0-15.2)
Magnesium (mg/L)	NA	5.6 ± 0.1 (5.3-5.9)	NA	11.6 ± 0.2 (11.3-12.0)
Conductivity (µS/cm)	NA	155.6 ± 1.4 (154.0-159.0)	NA	297 ± 2 (291-301)

[a] From United States Environmental Protection Agency (USEPA, 2002).
[b] NA = not applicable.

Table 3-6. Final total aquarium weights (FAW), estimated individual weight gain (IWG), specific growth rate (SGR), hepatosomatic indices (HSI), Fulton condition indices (K), and mortality rates of Rainbow Trout fed a reference (Ref) diet or diets with soybean meal and increasing levels of genistein at 30.6 (NoGen), 873 (LoGen), and 2,010 µg/g (HiGen) for 112 days. Means ± standard deviations listed with range shown inside parenthesis (n = 6). Values not significantly different ($P \geq 0.05$) have the same letter for a given dependent variable. Parametric statistical tests were used on variables listed with letters a-c, while letters y-z indicate the use of nonparametric analyses.

Diet	**FAW (g)**	**IWG[a] (g)**	**SGR[b] (%)**	**HSI[c] (%)**	**K[d]**	**Mortality[e] (%)**
Ref	555 ± 34[f]	9.5 ± 0.6 a	4.00 ± 0.06 a	1.44 ± 0.05 z	1.15 ± 0.02 a	4.8 ± 2.4 z
	(487-580)	(8.4-10.2)	(3.88-4.06)	(1.36-1.49)	(1.12-1.17)	(2.0-8.0)
NoGen	380 ± 20[f]	6.4 ± 0.2 b y	3.65 ± 0.03 b y	1.42 ± 0.05 z	1.08 ± 0.02 b z	4.0 ± 1.5 z
	(351-410)	(6.1-6.8)	(3.61-3.70)	(1.36-1.47)	(1.06-1.10)	(2.5-6.5)
LoGen	373 ± 12	6.2 ± 0.4 bc y	3.62 ± 0.05 bc y	1.41 ± 0.02 z	1.08 ± 0.01 b z	4.2 ± 2.0 z
	(356-390)	(5.8-6.8)	(3.57-3.70)	(1.39-1.44)	(1.07-1.09)	(2.0-6.0)
HiGen	353 ± 13[f]	5.8 ± 0.3 c z	3.56 ± 0.05 c z	1.41 ± 0.06 z	1.07 ± 0.02 b z	5.3 ± 1.3 z
	(335-376)	(5.4-6.3)	(3.49-3.64)	(1.34-1.50)	(1.03-1.08)	(4.0-7.0)

[a] IWG = final replicate mean weight – initial individual mean weight.
[b] SGR = 100 × [(ln final replicate mean weight – ln initial individual mean weight) / rearing days].
[c] HSI = 100 × (liver weight / total fish weight).
[d] $K = 10^5 \times$ [total fish weight / (total fish length)^3].
[e] Mortality = 100 × (total fish removed from death, disease, and/or abnormal behavior) / initial stocking rate.
[f] Indicates aquarium weight of 57 fish in one of the replicate aquaria, while remaining weights were of 58 total fish per aquarium.

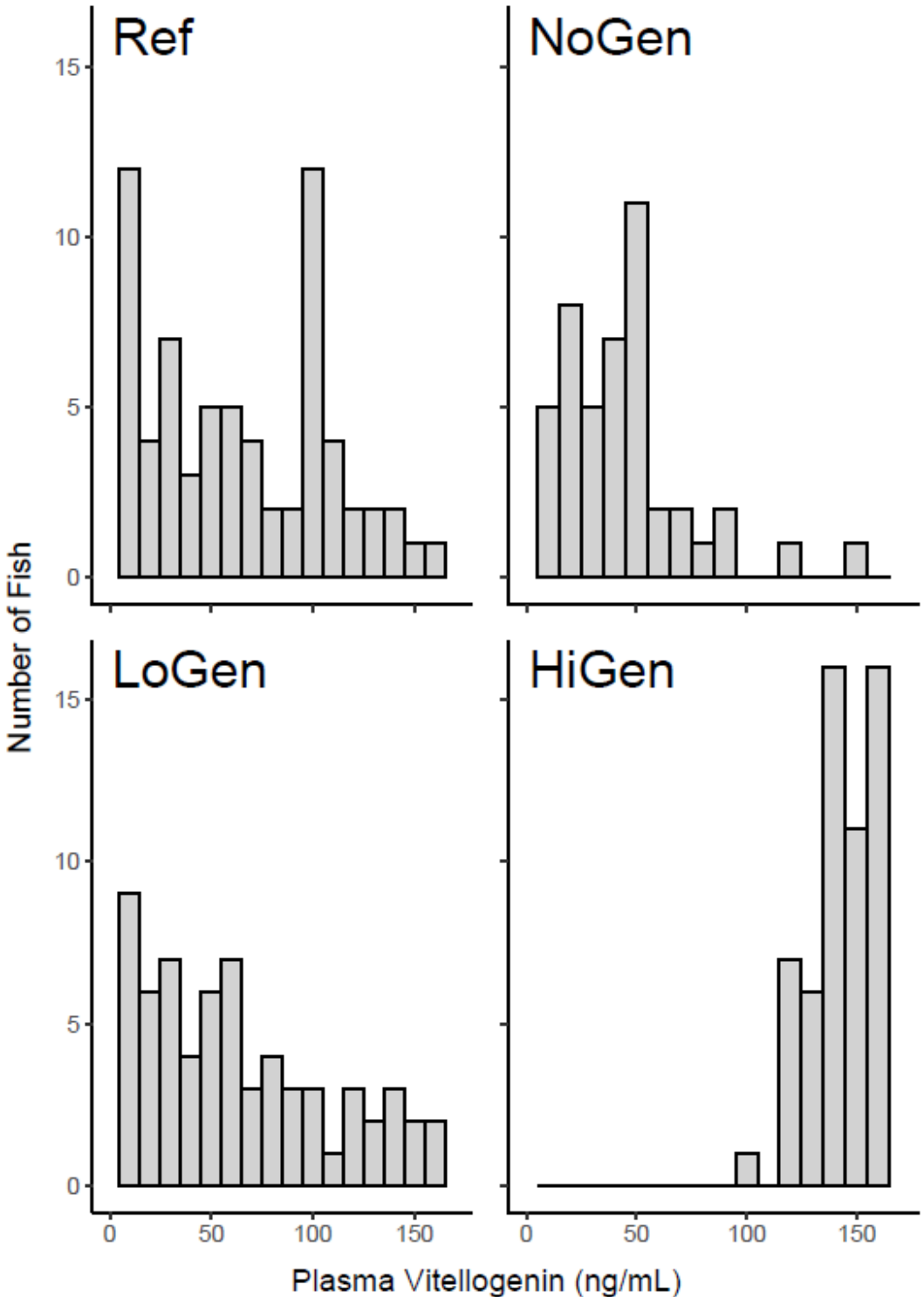

Figure 3-1. Number of fish fed a reference (Ref) diet or diets with increasing levels of genistein at 30.6 (NoGen), 873 (LoGen), and 2,010 µg/g (HiGen) falling within ranges of plasma vitellogenin.

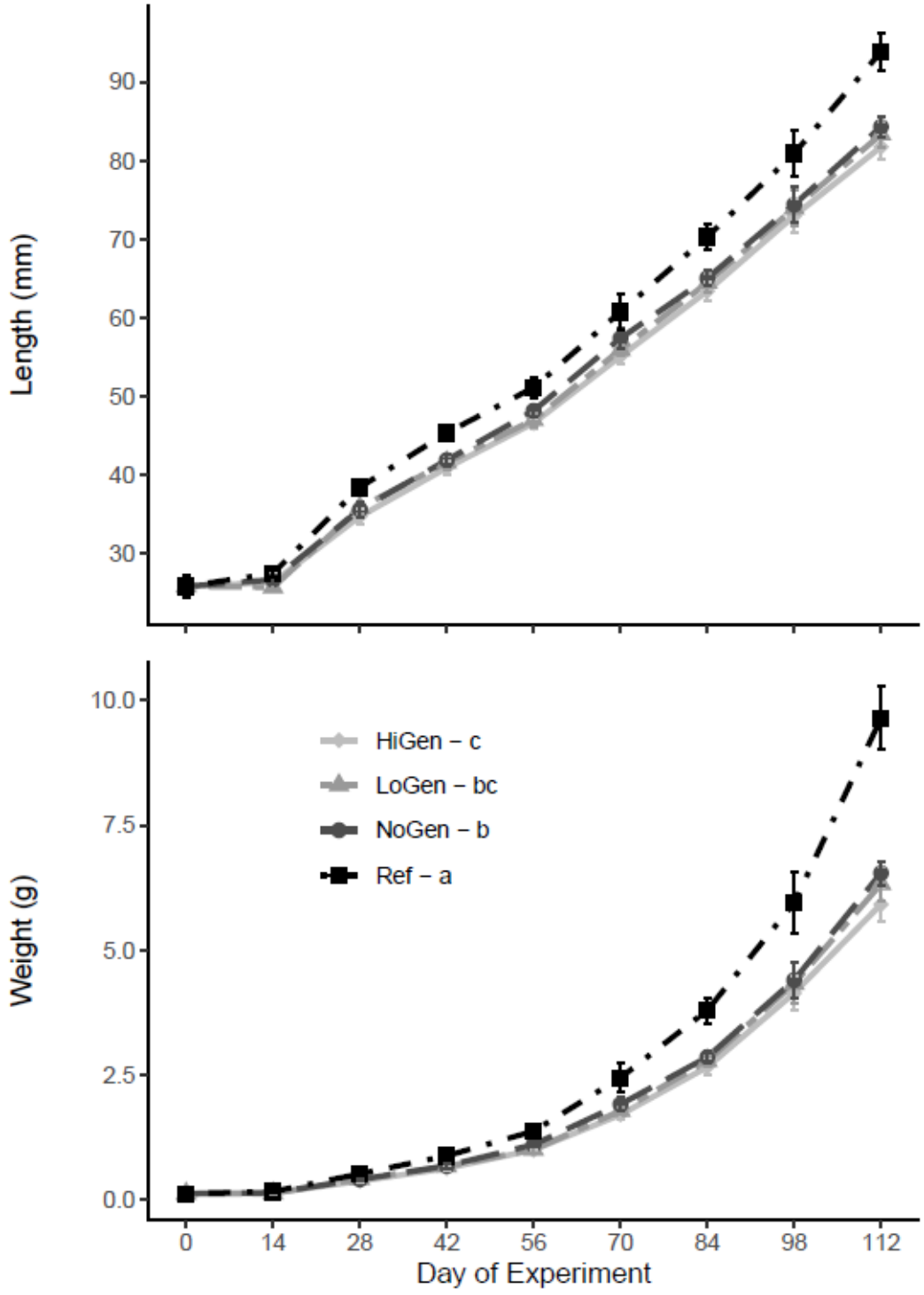

Figure 3-2. Replicate mean individual total length and weight growth patterns (mean ± standard deviation) of Rainbow Trout fed a reference (Ref; black squares, dot-dash) diet or diets with increasing levels of genistein at 30.6 (NoGen; dark grey circles, long dash), 873 (LoGen; medium grey triangles, short dash-long dash), and 2,010 µg/g (HiGen; light grey diamonds, solid). Growth curves not significantly different ($P \geq 0.05$) have the same letter listed in the legend.

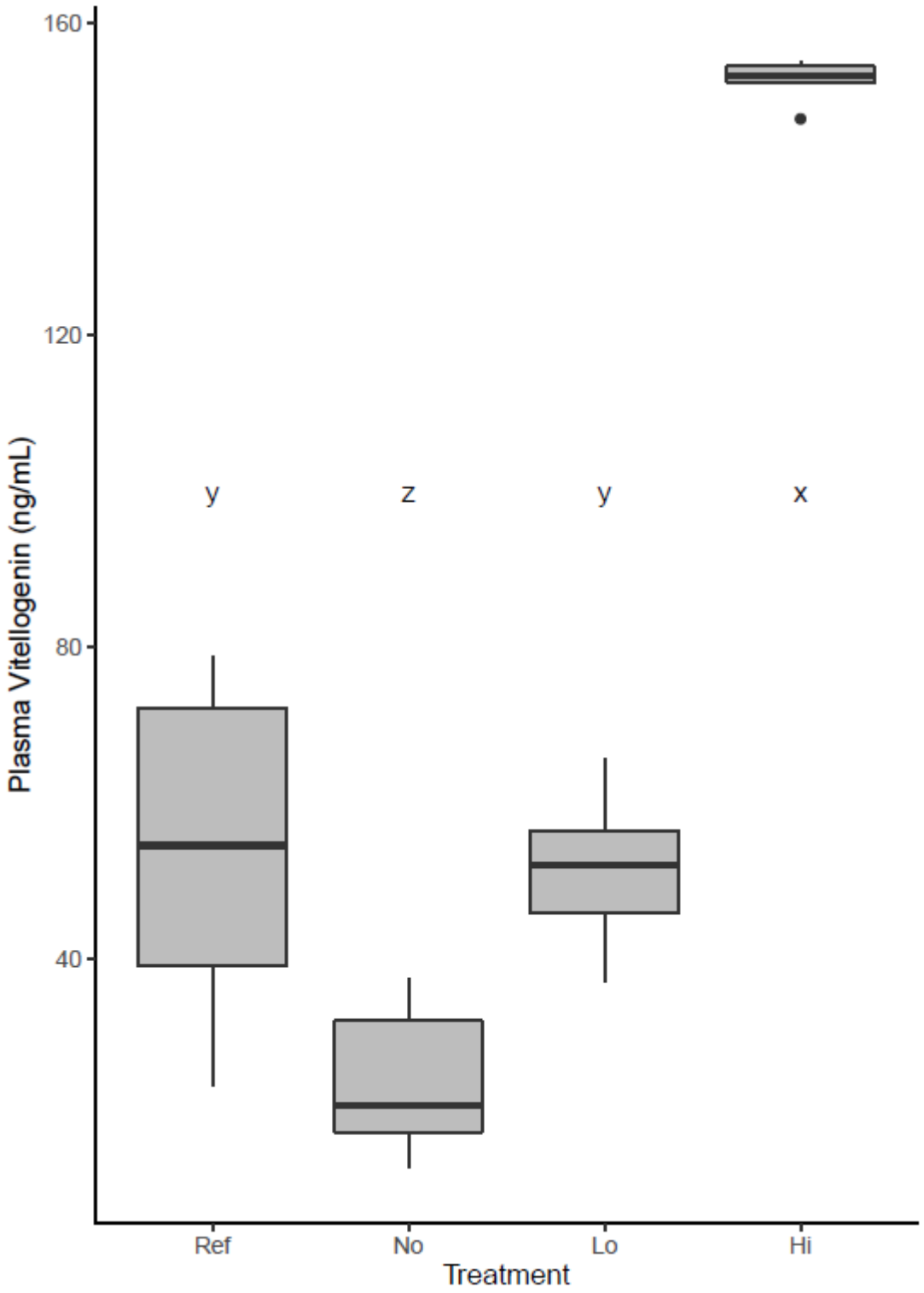

Figure 3-3. Plasma vitellogenin levels of Rainbow Trout fed a reference (Ref) diet or diets with increasing levels of genistein at 30.6 (NoGen), 873 (LoGen), and 2,010 µg/g (HiGen). Treatments not significantly different ($P \geq 0.05$), based on nonparametric methods, have the same letter.

CHAPTER 4. FUGACITY MODEL ESTIMATES OF GENISTEIN IN A LARVAL RAINBOW TROUT AND CULTURE SYSTEM

Introduction

As discussed previously in Chapter 2 of this dissertation, exposure to dietary and waterborne sources of isoflavones, and genistein particular, may induce a wide variety of sublethal effects on finfish species. Similarly, the results from the feeding trial in Chapter 3 demonstrated the physiological effects of dietary genistein on cultured Rainbow Trout (*Oncorhynchus mykiss*) larvae. While such research provided valuable insight into exposure effects, limited information exists for the genistein concentrations found in cultured finfish and distribution of genistein within fish tissues and excretory products. Only a few published studies have determined the genistein levels within different tissues of finfish exposed to dietary genistein (D'Souza et al., 2005; Gontier-Latonnelle et al., 2007). Such information would provide insight into biological processes involved within fish to incorporate and/or excrete these chemical compounds along with potential ontogenetic changes occurring during development. Several studies have presented information on the effects of genistein on digestive enzymes, enzymatic activities, and detoxification processes such as glucuronidation (Yokota et al., 2002; Gu et al., 2015; Olsvik et al., 2017); however, these studies utilized juvenile to adult fish, which do not account for the rapid ontogenetic changes that occur in larval fishes, which may be affected by genistein. Likewise, limited information exists on the potential for further exposure resulting from consumption of food items produced from cultured species or the use of their byproducts for other purposes. One study did determine genistein concentrations within Rainbow Trout fish fillets following exposure to genistein

(D'Souza et al., 2005), but did not provide much insight into potential exposure and possible effects on consumers.

Additionally, one exposure source of phytoestrogens currently not considered would be effluent from aquaculture facilities. While research has been performed on the negative environmental effects of aquaculture effluent, much of this research has been directed at various chemicals used for culturing practices such as antibiotics, antifoulants, and disinfectants along with nutrients (i.e., primarily eutrophication from nitrogen and phosphorus) (Dauda et al., 2019; Ahmad et al., 2021, 2022). However, as the aquaculture industry continues to grow and new innovations allow increased production yields, more emphasis and research is beginning to focus on newly found compounds that may be considered contaminants of emerging concern (CECs) (Ahmad et al., 2022). This concern, along with feed being reported as a major source of waste occurring within aquaculture systems (Dauda et al., 2019), indicates that compound leachates found within feeds and excretory products could prove to be a major source of environmental contamination. As such, genistein and other phytoestrogens could prove to be a potential CEC if found to occur in concentrations in waste products and/or effluents similar to those of other effluent sources. However, there remains scarce literature involving such research on aquaculture effluents and no published study has looked at genistein as a potential contaminant from aquaculture sources to my knowledge.

Along with the direct effects on cultured fish, phytoestrogens have been found to enter surface waters via several different sources including industrial, municipal, and agricultural non-point effluents (Lundgren & Novak, 2009; Kolpin et al., 2010; Rearick et al., 2014). While research has detected the presence of phytoestrogens within effluents

from these sources, the concentration threshold at which negative effects may occur in exposed wild fish populations has not been published (Lundgren & Novak, 2009; Rearick et al., 2014). However, some research has suggested a threshold of 1 µg/L total phytoestrogens as biologically significant and may result in disruptive effects upon fish (Lundgren & Novak, 2009; Kolpin et al., 2010; Rearick et al., 2014).

The development and application of mathematical models for research and regulation have been proposed and investigated to assess chemical fate and transport for such purposes as pollution control, prediction of impact scenarios, and investigations of transport, distribution, and fate of chemicals in the environment (Henderson et al., 2001; Hu et al., 2017). Among such models, fugacity models are used and based on thermodynamics equilibrium and mass balance principles. The fugacity concept has been successfully applied to estimate chemical fates on different physio-geographic regions and scales (i.e., ecosystem, biome, to global) and has proved a useful decision-support tool for determining regulations in environmental management and contamination control (Henderson et al., 2001; Wang et al., 2020; Chen et al., 2021). The following paragraphs on fugacity models are meant to provide a brief overview on the general development and use of fugacity models with primary importance being placed on the phases and processes necessary to develop a model for this dissertation research. For a more comprehensive review please read Mackay (2001) with emphasis on Chapters 1 and 5.

The concept of fugacity, describing the “fleeing” or “escaping” tendency of a chemical, was first introduced in 1901 by G. N. Lewis, but the idea did not receive much attention or use until the past three decades when it’s applicability for environmental modelling and chemical partitioning became more apparent (Jørgensen, 1990; Mackay,

2001; Newman & Unger, 2003). Fugacity is based on the laws of thermodynamics where a system consisting of different phases seeks to reach equilibrium between those phases following a chemical introduction. This occurs via the chemical potential of a system where it seeks to reach equilibrium using a minimum amount of free energy (Mackay, 2001). The underlying principle of phase equilibrium thermodynamics is that a chemical will seek to establish equal chemical potentials among all phases with the net diffusion flux occurring from high to low chemical potentials (Mackay, 2001). However, the use of chemical potentials for concentration determinations of various phases has several complications including, nonlinear relationships of chemical potential, concentration, and the inability to absolutely measure chemical potential (Mackay, 2001). The use of fugacity overcomes these complications by its identical nature to partial pressure in ideal gases that provides a logarithmic relationship with chemical potential (Mackay, 2001). Therefore, a linear or nearly linear relationship to concentration occurs allowing for the determination of absolute values (Mackay, 2001). Likewise, an advantage to using the fugacity approach over other chemical fate modelling techniques is the use of the same measurement units for all compartments derived from the formulas needed for such models (Newman & Unger, 2003).

Level I and II fugacity models are useful for determination of processes that control chemical fate and approximate estimations of chemical concentrations within different environmental compartments (Yenigun & Sohtorik, 1995) and are usually based on steady-state assumptions with equilibrium existing within each compartment allowing for the use of a common fugacity approach (Mackay & Paterson, 1991; Hu et al., 2017). Such an approach provides fairly simple, straightforward calculations; however, the

model assumptions have been reported to not simulate the dynamic processes that lead to steady-state within the environment (Hu et al., 2017). Aquaculture systems, specifically recirculating or flow-through systems, provide relatively stable systems compared to the dynamic processes and interactions that occur within a natural environment. Thus, these systems may provide a more stable environment that meets such fugacity assumptions allowing for the use of Level II or III models. Level III models have been used with several different organic chemicals with success in providing information on relative importance of environmental fate processes and/or concentration estimates within compartments (Mackay & Paterson, 1991; Chen et al., 2021). Likewise, Level III calculations can be used to address the assumption of equilibrium among environmental media/phases (i.e., using a common fugacity for calculations) needed to perform Level II calculations, which is rarely the case for most systems and environments (Jørgensen & Bendoricchio, 2001; Mackay, 2001). However, it is suggested by Mackay (2001) to start with a simple model and build complexity into it only when necessary and justified, for which the use of a Level III calculation will greatly increase the need for additional data and analyses to determine the transfer between phases (i.e., no equilibrium) (Jørgensen & Bendoricchio, 2001). Jørgensen (1990) also indicated that Levels I and II tend to be sufficient in most cases. Therefore, Level I and II fugacity modelling performed on a highly controlled system (i.e., flow-through aquaculture system lacking the normal complexity associated with natural systems) may be justified and thus, studied below.

To determine the distribution and fate of genistein within an aquaculture system and potential pathways (i.e., effluent, unused feed, fecal material, etc.) out of this system, an experiment was conducted in a simulated microcosm system with replicated

microcosms. Three-compartment microcosms consisting of air, water, and Rainbow Trout were established to simulate a flow-through aquaculture system and multi-compartment fugacity models were developed and used to predict distribution and bulk compartment concentrations of genistein. These predicted values were compared against measured concentrations of genistein in the different compartments of the microcosm system. This experiment provides, to my knowledge, a new and unused approach of fugacity modelling for determining dietary chemical fate and levels within an aquaculture system. Similarly, this experiment provides empirical data on the partitioning and concentrations of genistein found within the proposed microcosm system providing evidence of potential environmental contamination from aquaculture effluents. Therefore, the objectives of this study were to 1) determine and compare genistein concentrations found in whole fish, liver, fecal, and water samples, 2) develop Level I and II fugacity models to determine chemical partitioning and estimated genistein concentrations within each model compartment, and 3) determine model validity by comparing model estimates to empirical data for the fish and water compartment of the model.

Methods and materials

Experimental diets and fish

Experimental diets and larval Rainbow Trout used in this study were those described in "*Chapter 3, Methods and materials, Experimental diets and fish*" section. However due to financial and laboratory constraints, only chemical analyses obtained for the Ref, NoGen, and HiGen treatments were used for statistical analyses and modelling for this study. Therefore, the two experimental diets used in this study were formulated with additions of commercial SBM at 20 g/100 g diet and levels of genistein at 0.0

(NoGen) and 0.249 g/100g (HiGen). A reference diet (Ref) containing 20 g/100 g of fish meal with no SBM or genistein was also formulated for comparative purposes (Table 3-1). For detailed information on processing and analysis of experimental diets and on the culture, handling, acclimation, stocking, and study protocols of larval Rainbow Trout used in this study, see "*Chapter 3, Methods and materials, Experimental diets and fish*" section above.

Length and weight data of individual fish and tank weights were the same as those measured and used in the previous chapter; however, additional measurements were taken to determine fish volumes in each microcosm necessary for the fugacity models. For clarity on this sampling procedure, all steps will be described here including those used in "*Chapter 3, Methods and materials, Experimental diets and fish*" for length and weight data. These data were acquired as follows: 1) all fish were netted from a microcosm and placed in a plastic container filled with culture water from the microcosm, 2) a subsample were removed and placed in a separate plastic container for individual length and weight measurements, 3) remaining tank fish were poured into a large mesh net suspended over another culture-water filled plastic container, 4) the bottom of the net was blotted with paper towel to remove access water and the fish transferred to an appropriate-sized, tared graduated cylinder containing a known amount of culture water, 5) the graduate cylinder was then weighed to determine tank weight (± 1 g) and the volume displacement (± 1-5 mL) recorded, and 6) the fish were returned to their corresponding microcosm by inverting the graduated cylinder and rinsing it with culture water. Returned fish were then visually observed for any potential negative effects that may have occurred from handling (loss of equilibrium, erratic or abnormal

swimming behavior, etc.) and were replaced by subsampled fish of approximately the same size if such effects were seen. Fish volumes were measured every 14 days until the 56 DOE and then suspended to reduce potential stress until the final sampling period at 112 DOE. Subsampled fish from step two mentioned above were then processed as follows: 1) fish were euthanized, 2) total lengths (± 0.5 mm) were measured with a miniature measuring board, 3) fish were blotted with laboratory tissue paper to remove excess water and placed in a tared weighing dish to measure individual weights (± 1 mg), 4) all subsampled fish were then transferred to an appropriate-sized, tared graduated cylinder containing a known amount water, and 5) the graduate cylinder was then weighed to determine total subsample weight (± 0.01 g) and volume displacement (± 1 mL). All growth and physiological parameters associated length and weight data, along with mortalities rates, are described and reported in "*Chapter 3, Methods and materials, Experimental diets and fish*".

To acquire empirical data for comparison to fugacity model results, a variety of biological data was collected over the duration of this study. Pooled liver samples were collected at the mid- (56 DOE) and end-points (112 DOE) of the feeding trial by euthanizing a subsample of fish, extracting liver tissue, collecting and weighing each liver (±1 mg) into a tared, Nunc cryotube (340711, Roskilde, Denmark), and storing samples at -80°C until analysis to determine genistein concentrations and hepatosomatic indices (HSI) was performed; 3 pooled liver samples consisting of 3 sampled fish each were collected per replicate aquaria at both sampling periods. Pooled fecal samples were collected one week after the mid- (63 DOE) and end-points (119 DOE) of the feeding trial and within 24 h of the previous feeding by euthanizing a subsample of fish, gently

squeezing the lower abdomen just prior to the vent to expel fecal material, collecting and weighing each amount of fecal material into a tared, Nunc cryovial, and storing samples at -80°C until analysis to determine genistein concentrations was performed. One pooled fecal sample consisting of 8 sampled fish and 2 pooled fecal samples consisting of 4 fish each were collected per replicate aquaria at 63 and 119 DOE, respectively, to ensure enough fecal material for analysis. Due to the small size of larvae, pooled whole-body fish tissue samples were collected at 0, 56, and 112 DOE by euthanizing a subsample of fish, collecting larvae/fish into Nunc cryovial (363452) or 50 mL centrifuge tubes (14-955-240, Fisher Scientific, Pittsburgh, Pennsylvania) and storing samples at -80°C until analyzed for genistein concentrations and crude lipid content by the USGS, Columbia Environmental Research Center (CERC, Columbia, Missouri). All fish euthanizations that occurred within this study were performed with a lethal dose of tricaine methanesulfonate (Tricaine-S, Western Chemical, Inc., Ferndale, Washington). Pooled liver samples, fecal samples, and fish tissue samples were analyzed by CERC for genistein concentrations using mass spectrometric methods modified from Chang et al. (2000). For a detailed description of CERC analysis of these biological samples, see Appendix C. Likewise, CERC also assayed total crude lipid content of fish tissue samples; for a detailed description of this analysis see Appendix D. This study was performed in compliance with the South Dakota State University Institutional Animal Care and Use Committee (18-080A).

Microcosm and Culture System

A schematic diagram of the water-fish-air microcosm consisting of a glass aquarium measuring 75 x 30 x 30.5 cm (68.6 L capacity) stocked with Rainbow Trout

larvae is shown in Figure 4-1. For a full description of the culture system, reconstituted culture water, and water quality parameters with associated measurement methods, see "*Chapter 3, Methods and materials, Culture System*". To determine water volume levels necessary for fugacity modelling, a positive displacement water meter (Kent model C-700 Kent Industries Incorporated, Ocala, Florida), with resolution of 0.39 L (0.1 gallon), was used to measure the total amount of water needed to fill each microcosm to the standpipe overflow. Volume levels for each microcosm are listed in Appendix E.

To determine genistein concentrations within the culture water of each microcosm, 200 mL of water was collected from each microcosm as follows: 1) flow to all microcosms was stopped prior to sampling to reduce dilution in later sampled microcosms, 2) acid-cleaned, 100 mL volumetric pipettes were used to collected water for each treatment, 3) pipettes were inserted into a glass tube with nylon screening at the bottom to reduce larval impingement, 4) two 100 mL samples (i.e., 200 mL total) were collected into a sterile 200 mL glass amber jar with duplicate samples collected for each microcosm, 5) flow was re-established after all microcosms were sampled, and 6) samples were stored frozen from -20 to 0°C until they underwent analysis. Samples were collected at the mid- (56 DOE) and end-points (112 DOE) of the study, along with samples collected from the water supply tank at 112 DOE to ensure no background genistein concentrations from the water source. While collected in duplicate, only one sample per microcosm was analyzed for genistein concentrations by USGS, Organic Geochemistry Research Laboratory (OGRL, Lawrence, Kansas) with the remaining sample remaining in storage for further analysis if necessary. A detailed description of the water sample analysis can be found in Appendix F.

Statistical Analyses

All statistical analyses were performed using R (version 4.0.2) with the RStudio interface. The packages *asbio*, *rstatix*, *agricolae*, *nortest*, *statmod*, *ggplot2*, *gridExtra*, and *grid* were used for statistical analysis and graphics design; all statistical analyses were performed with $\alpha = 0.05$. All data used in statistical analyses underwent an initial test for normality using the Shapiro-Wilk normality test. If the test indicated data was not normally distributed ($P < 0.05$), the metric was analyzed with a non-parametric, 2-way analysis of variance type test based on ranks (Brunner-Dette-Munk test [BDM]) with treatment and DOE as factors (Brunner et al., 1997; Friedrich et al., 2017). If significant differences were detected between treatment means in the BDM test, post-hoc pairwise-comparison (PWC) tests were applied separately at the mid- (56 or 63 DOE) and end-points (112 or 119 DOE) to assess treatment means. The PWC tests were performed in such a way as no comparable 2-way nonparametric, post-hoc test could be found for Program R. Additionally, all metrics not normally distributed were *natural log* + 1 transformed and re-evaluated for normality in an attempt to use parametric statistical tests; however, metrics remained not normally distributed after such transformations and resulted in the use of the non-parametric methods mentioned above.

Fugacity Modelling

Model Development

The models used in this study were developed using the fugacity approach for Level I and II calculations (Yenigun & Sohtorik, 1995; Mackay, 2001) and consisted of three major bulk compartments, air (*A*), water (*W*), and fish (*F*), each with a defined total volume (*V*). Each compartment consisted as a combination of sub-compartments of pure

and particle phases. This would include such factors as the water compartment containing "pure" water, suspended solids, fish metabolites, etc.; air compartment containing "pure" air and un-measured aerosols; and the fish compartment consisting of all parts (i.e., organs, skin, etc.) of the specimen at the given sampling time. A list of the compartments along with the associated *V* and *Z* values are listed in Table 4-1. The model is meant to be evaluative on a very small scale for that of hatcheries and aquaculture operations raising freshwater finfish when compared to other fugacity models such as those developed for large-scale areas and natural environments (Mackay & Paterson, 1991; Mackay, 2001).

It is necessary to describe the basic equations and calculations needed for fugacity modelling for concentration determinations of a chemical in various compartments. As mentioned earlier, a linear relationship exists at low concentrations between fugacity and the concentration of a chemical, making them proportional with the equation:

$$C = Zf$$

where *C* is the concentration (mol/m^3), *Z* is the proportionality constant (i.e., fugacity capacity, mol/m^3 Pa), and *f* is the fugacity (Pa) (Jørgensen, 1990; Mackay, 2001; Newman & Unger, 2003). It is necessary to determine the *Z* values within each compartment to assess the chemical partitioning among compartments. To accomplish this task, knowledge of partitioning coefficients (*K*) of a chemical between different compartments ($_1$ and $_2$) is needed and can be determined through the linear relationship of chemicals at low concentrations with the equation:

$$C_1/C_2 = K_{12}$$

therefore, by replacing C_1 and C_2 with corresponding *Zf* values and when f_1 equals f_2 (i.e., equilibrium), the following equation results (Jørgensen, 1990; Mackay, 2001):

$$K_{12} = Z_1/Z_2.$$

This relationship provides a method for estimating *Z* values (i.e., "half" partition coefficients) if one *Z* and *K* value is known (i.e., Z_1 equals $K_{12}Z_2$) (Jørgensen, 1990; Mackay, 2001). This type of relationship is depicted in Figure 4-2. The concept of *Z* is based on the ability of a phase to absorb or take up a given amount of chemical, similar to the concept of heat capacity (Mackay 2001). Phases with large *Z* values can absorb a greater amount of chemical while maintaining a low fugacity; therefore, a chemical will partition into and accumulate high concentrations within a compartment(s) with large *Z* values even at a low fugacity (Mackay, 2001).

Partition coefficients between numerous phases can be determined; however, the two of primary interest for most applications are air-water (K_{AW}) and octanol-water (K_{OW}). Pioneering work performed by W. Henry on air-water partitioning found that solubility in water was proportional to partial pressure (Henry, 1803; Mackay, 2001). Using this concept, Henry's law constant (*H*) for a chemical can be expressed as the ratio of its partial pressure in vapor (i.e., Pa) to the concentration in the liquid (i.e., water) and is usually expressed as a Pa m^3/mol (Mackay & Shiu, 1981). With a defined *H*, K_{AW} can then be calculated using the equation:

$$K_{AW} = H/RT = Z_A/Z_W$$

where *R* is the gas constant (8.314472 J/mol *K*) and *T* is temperature in Kelvin (°K). Based on this equation, *Z* values for air ($_A$) and water ($_W$) can be expressed as *1/RT* and *1/H*, respectively (Jørgensen, 1990; Mackay & Shiu, 1981; Mackay, 2001). Along with K_{AW}, the K_{OW} is one of the most important chemical descriptors of chemical behavior within an environment by indicating the hydrophobicity (i.e., tendency to "hate" or

partition out of water) of that chemical (Mackay, 2001). The use of 1-octanol for determining chemical partitioning gained popularity due to its similar carbon to oxygen ratio as lipids, tested correlations with many bio-chemical phenomena, very low solubility in water, and the relative availability of its pure form for testing (Mackay, 2001). The properties of octanol simulate those of lipids, which acts as the primary sorbing/dissolving medium of hydrophobic organic chemicals in fish (Mackay, 2001). Due to these similarities, a consistent relationship exists between K_{OW} and the lipid-water partitioning coefficient (K_{LW}) with both assumed to be equal under most circumstances (Mackay, 2001). Therefore, this relationship allows for determination of the fish-water partitioning coefficient (K_{FW}) and Z value for fish ($_F$) by using the following equation as found in Hu et al. (2017):

$$Z_F = L_F K_{OW} \rho_F / H$$

where L_F is the fish lipid content (volume fraction) and ρ_F is the density (kg/m^3) of fish. Known L_F of the species and life stage will provide more accurate results compared to using estimated lipid values (Mackay, 2001). Consequently, crude lipid levels from sampled fish were determined for each microcosm. Likewise, fish densities for each microcosm were calculated from total fish mass and volume measurements collected at the mid- and end-points of the study. These methods are mentioned above along with those used to determine crude lipid levels of sampled fish in each microcosm.

Due to the use of Level I and II fugacity models, it is assumed that genistein reached equilibrium within each compartment allowing for the use of a common fugacity for calculations. This assumption implies that the time reach equilibrium within compartments is shorter than the time needed between compartments (Mackay &

Paterson, 1991). Likewise, it is assumed that all *V* and *Z* values were constant with time and that growth (i.e., volume increase) of fish to be negligible. As such, several models were performed using genistein inclusion levels fed (i.e., total genistein fed over 13 days) two weeks prior to the mid- and end-point measurements allowing for such assumptions to be held as it provided the least amount of variability due to changing feeding rations and fish volumes associated with growth as both are necessary parts of an aquaculture feeding trial. Similarly, another study analyzing Level III fugacity models of ethiprole found that the chemical reach steady-state equilibrium at 14 d (Hu et al., 2017), which is similar to the 2 week feeding schedules used to adjust feeding rations and thereby, chemical input. However, to also look at the potential effects of chemical input, total amounts of genistein fed from study start to 56 DOE and to 112 DOE were used in both the Level I and II fugacity models, while holding all other parameters the same. Most model parameters for Level II calculations were garnered from the literature, including environmental and physiochemical parameters, and are listed in Table 4-2. Also, all associated degradation rates and corresponding *D* values needed for Level II calculations are listed in Table 4-1.

At this point, all the necessary chemical (i.e., partitioning coefficients, *H* value, etc.) and environmental (i.e., compartment volumes, lipid levels, etc.) information exist for Level I calculations to be performed to determine how partitioning of a chemical occurs between compartments along with estimated concentration levels (Jørgensen, 1990; Jørgensen & Bendoricchio, 2001; Mackay, 2001). Level I calculations were used to estimate genistein concentrations found within each compartment of the proposed fugacity model using basic algebraic equations listed in Table 4-3. These calculations

were performed for each microcosm using the corresponding data collected at the mid- and end- point sampling periods for each microcosm. These estimated results were then compared against the empirical data collected at the corresponding sample point to evaluate model performance.

To address the potential flux a chemical entering and/or exiting a microcosm along with degrading chemical reactions, Level II calculations are needed (Jørgensen & Bendoricchio, 2001). With such calculations, the chemical is viewed as a rate flowing into and out of the system at a set rate (mol/hr) unlike a total amount of chemical added to the system as in Level I calculations. To determine the amount of chemical entering the system for Level II calculations, it was necessary to determine the rate of genistein entering each microcosm via the diet (i.e., daily feeding rations [g/d]). As mentioned earlier, data from 2 weeks prior to sampling periods were used, which coincided with the use of set feeding rations to each microcosm and total amount fed to each sampling point. Due to the need to change feeding rations within this time period and to determine the necessary influx rate of genistein (mol/hr), the following steps were performed: 1) the total amount of food was determined for the feeding period then divided by the total number of h occurring during days fed, providing g diet/hr estimates for each microcosm; 2) the number of g diet/hr was multiplied by the corresponding treatment concentration of genistein (µg genistein/g diet) found in each diet as listed in Table 3-1 providing µg genistein/hr, which was converted to g genistein/hr; and 3) g genistein/hr was converted to mol/hr using the molar mass of genistein found in Table 4-2. Only the number of days fish were actually fed (13 days) was used to determine influx rates for the two weeks prior to sampling (L2W), the sample day was not included within the time period for

calculations. Similarly, data for influx rates occurring for the total amount of genistein fed (TGA) up to the mid- and end-points excluded sampling days during those time periods leaving total days fed at 52 and 104 days, respectively.

Along with the influx rate, several other processes are necessary to consider with Level II fugacity calculations. The first process is advection and is defined in Mackay (2001) as "the directed movement of chemical by virtue of its presence in a medium that happens to be flowing" with the rate of advection (N_A) being calculated as:

$$N_A = GC$$

where, G is the advecting medium flowrate (m^3/h) and C is the concentration of a chemical in that medium (mol/m^3). By replacing C with the corresponding Zf term and then adding inflows of chemical from each individual ($_i$) phase, the total influx (I) of chemical into the system and/or the overall constant f can be calculated with equation:

$$I = f(\Sigma G_i Z_i) \text{ or } f = I/(\Sigma G_i Z_i),$$

which then allows for chemical concentrations in each phase to be calculated (Mackay 2001). Due to their frequent use in calculations, the transport rates listed as GZ are normally designated as D_A values (Mackay, 2001; Newman & Unger, 2003). Due to the controlled nature of the experimental system whereby, genistein only entered the system via the diet and by no other means (i.e., water inflow, aerosol depuration, background burdens within stocked fish, etc.), advection for each compartment was assumed to be 0.

The second process to consider is degrading reactions, which are defined as processes that change the overall nature (i.e., structure, formula, etc.) of a chemical (Mackay, 2001). Information on biodegradation, photolysis, and water degradation were considered when determining D values of reactions (D_R) for the current fugacity model

(Table 4-2). When possible, it is preferable that first-order kinetic expressions of reactions be incorporated by using the following basic rate of reaction (N_R) equation:

$$N_R = VCk$$

where V is volume of the phase (m^3), C is concentration as mentioned earlier, and k is the first-order rate constant having units of reciprocal time (e.g., h^{-1}) (Jørgensen, 1990; Mackay, 2001). The concept of reciprocal time for k is difficult to conceptualize; therefore, it is better to use half-lives ($\tau_{1/2}$) which depict the time required for a chemical concentration to decrease by half from its original concentration and is related to k as

$$\tau_{1/2} = 0.693/k.$$

It is also beneficial to use first-order kinetics as the rate constants for several different reactions occurring within the same phase as a total rate constant for reaction can be calculated by adding all the individual rate constants together (Mackay, 2001). Like D_A values, the Zf term can be substituted to create the following equation

$$N_R = VZfk = D_R f$$

where D_R is VZk and maintains the same unit (mol/m^3 Pa) as that of D_A. Since the D_R values are considered under first-order kinetics, each reaction is assigned a D value and all D values can then be added to determine a total D_R value, which can then be used to determine the emission (E) rate of the system using equation

$$E = f\Sigma D_R \text{ or } f = E/\Sigma D_R.$$

After both D_A and D_R values have been determined, the total influx (I) to the system can be determined from the equation

$$I = E + \Sigma G_i Z_i + \Sigma V_i Z_i k_i = E + \Sigma D_A + \Sigma D_R$$

from which the system fugacity, compartment concentrations, and chemical partitioning can be determined (Mackay, 2001). Values for D_R were calculated for each compartment using microcosm specific data in Appendix E and the formulas listed in Table 4-4. Similar to Level I calculations, data corresponding to each microcosm was used in Level II calculations and compared against corresponding empirical data to evaluate model performance. Additionally, the calculations and their associated steps necessary for Level II models are presented in Table 4-4.

Model Evaluation

A valid model must be able to produce estimates that are both adequate and satisfactory, taking into account the concepts of model variance and bias. To determine if the proposed models are valid, statistical analyses were performed to determine if the estimated concentrations of genistein in each compartment were similar to empirically derived values. The analysis used here to evaluate model performance was percent bias (PBIAS) and is calculated as follows:

$$PBIAS = \frac{\sum_{i=1}^{n}(Obs_i - Sim_i)}{\sum_{i=1}^{n}(Obs_i)} \times 100\%$$

where Obs_i and Sim_i are the i empirical and estimated data from the study and model, respectively; n is the total amount of observations (Hu et al., 2017; Chen et al., 2021). This calculation determines goodness-of-fit by measuring the average tendency of estimated data to be greater or less than its associated empirical data with calculations of PBIAS falling within the range of -70% to 70% deemed satisfactory; 0% indicates no bias (Hu et al., 2017; Chen et al., 2021). This calculation also indicates under- or over-prediction of model estimates when positive or negative values occur, respectively (Hu et al., 2017; Chen et al., 2021). To determine potential modelling differences and

corresponding model performance of treatments, PBIAS was calculated for empirical and estimated pairs of each treatment at the mid- and end-points of the study. Based on the literature, PBIAS appeared to be used on a regular basis for determining the validity of fugacity models compared to other validation methods such as root square mean error and was therefore selected for use with the current study.

Results

To determine the fate of genistein within the culture system at early life stages, the data was collected and analyzed, fugacity models developed, and comparisons between model estimates and empirical data performed at both the mid- and end-point of the feeding trial. Genistein concentrations analyzed in whole-body, fecal, liver, and water samples displayed a very wide range of variability within replicates of the NoGen and HiGen treatments (Table 4-5). This variability was not unexpected due to the nature of the samples and analytical procedures used. However, even with such a large range and high standard deviations, statistical analyses found significant differences for four different types of samples. A similar trend was seen for the samples of whole-body, fecal, and liver with significant differences occurring for both the treatment and DOE levels and post-hoc tests indicating significant differences between all treatments. For whole-body, the BDM test found significant differences for both factors of treatment ($P = 2.69 \times 10^{-15}$) and DOE ($P = 0.0397$), while the interaction term was not statistically significant ($P = 0.293$). The PWC tests indicated that all treatments were significantly different from one another at both the mid- ($P = 0.0065$) and end-points ($P = 0.0065$) (Table 4-5). For genistein concentrations found in collected fecal material, the BDM test indicated significant differences for both treatment ($P = 2.42 \times 10^{-12}$) and DOE ($P = 0.000774$),

while the interaction term was not statistically significant ($P = 0.103$). The PWC tests indicated that all treatments were significantly different from one another for both the mid- ($P < 0.0094$) and end-points ($P = 0.0065$) (Table 4-5). For genistein concentrations found in the liver samples, the BDM test indicated significant differences for both treatment ($P = 4.13 \times 10^{-14}$) and DOE ($P = 0.00336$), while the interaction term was not statistically significant ($P = 0.914$). The PWC tests indicated that all treatments were significantly different from one another at both the mid- ($P < 0.0087$) and end-points ($P = 0.0087$) (Table 4-5). In a similar fashion, the BDM test for genistein concentrations in the culture water indicated significant differences for treatment levels ($P = 1.16 \times 10^{-8}$) and DOE ($P = 0.00536$) but no statistical difference of the interaction term ($P = 0.0704$). Unlike previous parameters, PWC tests indicated the reference treatment was statistically lower at both the mid- ($P < 0.012$) and end-points ($P = 0.011$), while no statistical differences occurred between the NoGen and HiGen treatments at either point of the study ($P > 0.132$) (Table 4-5).

Model evaluation of each treatment at the mid- and end-points using PBIAS indicated a wide range of model performance. When analyzing Level I models, total amounts of genistein included did have an apparent effect upon model performance based on genistein amounts from the L2W data versus those the TGA dataset for the mid- and end-points (Figure 4-3). Model performance for genistein estimates at mid- and end-points within fish and water using L2W data indicated that PBIAS values were within the acceptable range of -70 to 70% for the Ref (27 to 70%) and HiGen (-15 to -18%) treatment models, except for the HiGen treatment at the mid-point (-816%). Within these acceptable models, all Ref and HiGen treatment models under- and overestimated the

associated empirical data, respectively. All models for the NoGen treatment were >70% from 71 to 96% indicating underestimation with poor model performance. In contrast, models using TGA data indicated acceptable model performance with PBIAS between -70 and 70% for all models of the NoGen (6 to 61%) treatment, except water at the end-point (80%), and the Ref treatment for fish (-39%) and water (-61%) at the mid- and end-point, respectively. Within these acceptable models, all Ref and NoGen treatment models over- and underestimated the associated empirical data, respectively. For the HiGen treatment, all models overestimated with poor model performance (<-211%).

In contrast to Level I model estimates, results from PBIAS analyses of all Level II models were >92%, indicating that model estimates were underestimated with poor model performance for both L2W and TGA data sets. The exceptions were estimates of genistein in water for the HiGen treatment at the mid-point with L2W and TGA values of PBIAS at 44% and 63%, respectively (Figure 4-4). Also, all models utilizing the TGA data performed poorer than their counterpart models using the L2W data set with PBIAS decreasing from 1 to 19%.

As noted earlier, the developed fugacity models included three compartments consisting of fish, water, and air; no results are presented for the air compartment as no empirical data was collected and was not of interest within the scope of this study. However, it was included within the models due to its potential effects upon calculation of the overall system fugacity and corresponding genistein concentrations in fish and water (Level I and II calculations) along with the fact photolysis rates were found within the literature (Level II calculations). In order to determine potential effects upon model performance, both Level I and II fugacity models were run with and without inclusion of

the air compartment and PBIAS was calculated for each. The results found a <1% difference in PBIAS calculations from all reported values shown in Figures 4-3 and 4-4. Because these results were for comparative purposes that did not result in any meaningful increase in goodness-of-fit, no additional results are reported or discussed in this chapter.

Discussion

Genistein concentrations in water

Results from this study provide evidence for the presence of genistein within the various compartments of an aquaculture system and the cultured fish themselves via dietary introduction. With regards to the water compartment within the microcosms, genistein concentrations were observed to increase with both dietary inclusion and over time. Such increases would be expected due to the increased dietary genistein concentrations. Likewise, increased concentrations of genistein from the mid- to end-points of the study are likely associated with the increased feeding rations needed for larger-sized Rainbow Trout resulting in higher levels of genistein introduced to the system. To my knowledge, no effort or study has been reported indicating potential genistein concentrations found within the water of aquaculture systems. However, several studies have determined genistein levels in various anthropogenically impacted surface waters; therefore, a comparison to the current genistein water concentrations would be beneficial. Industrial waste water effluents have been shown to contain levels of combined phytoestrogens from 1 ng/L to 250 µg/L (Lundgren & Novak, 2009; Rearick et al., 2014), with genistein being found in treated pulp mill effluent at 10.5 µg/L (Kiparissis et al., 2001), biodiesel processing wastewaters from 306-10,300 ng/L, human food production effluents from 71.4-151,000 ng/L, and waste water treatment plant

effluents from 32.6-158 ng/L (Lundgren & Novak, 2009). Similarly, genistein was found in surface waters of anthropogenically impacted lakes and streams. Two such impacted water bodies in Minnesota, Lake Vadnais and the Metro Plant effluent channel, were found to contain an average of 1.4 ± 0.5 ng/L genistein compared to no measurable levels found in a reference lake deemed relatively pristine (Rearick et al., 2014). Also, agricultural runoff into streams has shown maximum genistein concentrations at 8 ng/L from samples collected across Iowa (Kolpin et al., 2010) and 10.2-24.2 ng/L at different sampling sites for two Swiss rivers (Hoerger et al., 2009). In comparison, the mean genistein concentrations within the current study ranged from 779-2,488 ng/L for the NoGen treatment placing such water samples well above those measured in impacted surface waters and more in-line with concentrations found within industrial waste waters.

Due to these concentrations within the sampled microcosm waters, it is highly likely that water effluent produced from such flow-through aquaculture systems could become a potential source for genistein entering the environment. Such effluents could potentially be a source of vulnerability to wild populations of fish exposed to such levels of water-borne genistein as it reached levels deemed environmentally relevant (≥1,000 ng/L) (Lundgren & Novak, 2009; Kolpin et al., 2010; Rearick et al., 2014). Likewise, a study was performed on Fathead Minnows (larval and adult) to quantify mortality and physiological responses when exposed to several phytoestrogens (genistein, daidzein, and formononetin) both singly and in a mixture (Rearick et al., 2014). Results from the study found that survival was statistically lower for larvae exposed to water-borne genistein compared to the positive and negative controls, while subtle but not statistically significant results were observed for growth, latency, and escape velocity of adult

minnows (Rearick et al., 2014). In another study analyzing water-borne genistein, adult Japanese Medaka were exposed to increasing levels of genistein from 1-1,000 µg/L to evaluate potential impacts on gonad development and differentiation and expression of secondary sex characteristics (Kiparissis et al., 2003). While genistein did not affect mortality or spermatogenesis, other concentration dependent effects occurred such as delayed oocyte maturation, atretic oocytes, enlarged ovarian lumen, increased somatic stromal tissue, increased connective tissue and fibrosis around testicular lobes, and increased numbers of fish with low spermatozoa densities (Kiparissis et al., 2003). Likewise, intersex characteristics and an increased occurrence of males with feminine secondary sex characteristics occurred in fish exposed to 1,000 µg/L (Kiparissis et al., 2003). Along with these studies, several performed on Siamese Fighting Fish found that exposure to water-borne genistein levels from 1-1,000 µg/L resulted in significant decrease in duration of opercular displays and significantly lower sperm totals at 1ug/L genistein compared to fish treated with 1ug/L 17β-E2, but no significant effects were detected for reproductive behaviors or hormones (Clotfelter & Rodriguez, 2006; Stevenson et al., 2011; Brown et al., 2014). Based on these studies, it seems that wild fish populations exposed to potential effluent levels found in the NoGen treatment water could be negatively affected by water-borne genistein concentrations. Likewise, larval fish appear to have a high susceptibility to the reported genistein concentrations compared to adult life-stages; therefore, it becomes important to determine the potential effluent sources from aquaculture operations if located in areas where spawning is known to occur for wild populations. In addition, the findings from this study point to the possibility of genistein and other phytoestrogens as an additional food-borne contaminant

found in aquaculture effluents. Currently, a number of other CECs such as unused nutrients, antibiotics, antifoulants, and disinfectants found in aquaculture effluent have received attention due to contamination of receiving water bodies (Ahmad et al., 2021, 2022); thus inclusion of genistein and other phytoestrogens should be considered.

Genistein concentrations in and from fish

In consideration of genistein concentrations found in the cultured Rainbow Trout and their corresponding tissues and excretory products, the following discussion focuses on the incorporation of genistein into these areas. For the effects that fed genistein had on the morphology and physiology of the fed Rainbow Trout, see Chapter 3 of this dissertation above. Similar to the results for genistein concentrations in water, those found in the whole-body, fecal matter, and livers all indicated increasing concentrations of genistein corresponding to dietary levels. This indicated a straight-forward, dose-dependent relationship between dietary inclusion/uptake of genistein and incorporation into various Rainbow Trout tissues and excretory products. A similar pattern was detected in another study where Rainbow Trout were fed for 12 months exhibited a statistically significant, positive correlation between dietary levels of genistein (0, 500, 1,000, and 3,000 µg/g) and those found in sampled fish fillets after 6 and 12 months of feeding (D'Souza et al., 2005). The highest average genistein content in fillets was 1.46 µg/g (5.4 pmol/mg) in Rainbow Trout fed the highest level; however, the measured genistein concentrations were not statistically different (D'Souza et al., 2005). The authors indicated that increased genistein deposition within the tissues did not adversely affect flavor, color, or proximate composition of fillets and indicated genistein concentrations in the fillets were about $1/100^{th}$ lower than those found in commercial soy

foods (D'Souza et al., 2005). These results indicated lower amounts of genistein within the fish; however, only muscle tissue was analyzed, whereas whole fish samples including all organs (i.e., brain, liver, pancreas, etc.) were included for modelling purposes and thus, would be expected to have higher genistein concentrations due to the inclusion of all fatty tissues during analysis. Nonetheless, such concentrations of genistein in whole-body samples, does raise the question whether cultured fish flesh could be a dietary source of genistein to humans. Most consumption of genistein by humans occurs via soy-based food products and has been shown to have beneficial physiological effects (Delclos et al., 2001; Setchell, 2017). However, concerns toward potential adverse effects via maternal exposure associated with isoflavone consumption have been studied due to the interaction of genistein with estrogen receptors as a hormonally active chemical (Delclos et al., 2001). One study looked at the developmental effects on CD (Sprague-Dawley) rats (*Rattus rattus*) through dietary exposure of increasing levels of dietary genistein from 5-1250 µg/g fed to dams (Delclos et al., 2001). Results from the study indicated dams fed 250-1,250 µg/g treatments of dietary genistein produced negative effects in multiple estrogen-sensitive tissues of both male and female pups (Delclos et al., 2001). The authors suggested that a concentration of 500 µg/g was unlikely to be overly toxic to dams and their pups, but had probable effects on the reproductive system (Delclos et al., 2001). Based on these findings, the current concentrations of genistein in fillets reported in D'Souza et al. (2005) and whole-body samples (≤459 µg/g) at the end of this current study would be unlikely to contribute to any negative physiological effects upon maternal consumers and their offspring unless fed whole fish products from cultured fish receiving high (>2,000 ug/g) levels of dietary

genistein. However, the presence of genistein along with the overall total amount of phytoestrogens present in diets and subsequent fish tissues should not be completely dismissed due to potential physiological effects upon consumers.

Similar to genistein concentrations in water, significant differences occurred between the mid- and end-points for genistein concentrations in whole-body, liver, and fecal samples; however, unlike the water samples, decreases occurred in genistein concentrations over time for whole-body and liver samples, while fecal concentrations increased similar to water. Likewise, the overall percentage of genistein found between these different components depicts similar patterns. At the mid-point, whole-body sample percentages (calculated as mean whole-body minus mean liver concentration for each treatment divided by the total amount of genistein found in all three sample types) for NoGen and HiGen treatments ranged from 82.5-87.2% of the total genistein found in whole-body samples, while 12.0-17.2% were seen in fecal samples. At the end-point of the study, these percentages shifted to 23.3-49.1% of total genistein in whole-body samples and 50.3-76.5% in fecal samples. Such pattern shifts seem to suggest that as the cultured Rainbow Trout grew older, ontogenetic changes to the digestive tract resulted in lower amounts of genistein being absorbed (i.e., flesh and organs) and more excreted as fecal material. During this stage of ontogeny, larval fish experience a variety of dynamic and complex developmental processes of various organs with the functioning of digestive capacity dependent upon digestive system development (Sala et al., 2005; Gu et al., 2015). One study sought determine early developmental effects of isoflavones on larval Atlantic Salmon (*Salmo salar*), including digestive enzyme activities and intestinal morphology by feeding 1.5 g/kg genistein to first-feeding Atlantic Salmon and comparing

against controls (Gu et al., 2015). Results indicated that no apparent histology changes occurred in either the pyloric caeca or distal intestine of larvae fed for 98 days; however, maltase and trypsin activities of fish fed isoflavones were statistically lower and higher, respectively, than those of the control fish (Gu et al., 2015). Unfortunately, samples were not taken at differing time periods during this study to depict potential ontogenetic effects over time; however, changes associated with dietary genistein do show potential effects upon digestive processing of genistein within a salmonid species.

Another possible explanation for increased genistein excretion via the digestive tract would be increased glucuronidation activity of genistein in older Rainbow Trout. Glucuronidation has been deemed as an important detoxification and elimination mechanism of various lipophilic compounds including xenoestrogens and phytoestrogens (Yokota et al., 2002; Mackenzie et al., 2010). The process includes covalent addition of glucuronic acid to the nucleophilic atom (i.e., oxygen, nitrogen, sulfur, or carbon) on a lipophilic compound resulting in a charged, water-soluble glucuronide that facilitates excretion of the parent compound via excretory products (Mackenzie et al., 2010). In a study analyzing the disposition of genistein within various Rainbow Trout tissues, the authors found that glucuronidation was the most important pathway for biotransformation of genistein within Rainbow Trout (Gontier-Latonnelle et al., 2007). Along with that finding, another study found that glucuronidation within Common Carp (*Cyprinus carpio*) played an important role in detoxification of phytoestrogens, including genistein (Yokota et al. 2002). This study determined microsomal UDP-glucuronosyltransferase activities in the gill, kidney, hepatopancreas, and intestine tissues collected from 0.5, 1, 2, and 4 year old Common Carp and found significantly higher levels of glucuronidation in

the intestinal mucosa microsomes compared to the other tissues with a 2-fold higher activity than the hepatopancreas (Yokota et al., 2002). Likewise, genistein underwent the same degree of glucuronidation in the carp intestinal microsomes as that of rat liver microsomes and appeared to increase developmentally with age of the fish (Yokota et al., 2002). Such findings suggest that as the Rainbow Trout in this study aged and underwent digestive ontogeny, the digestive tract increased glucuronidation capacity and activities resulting in higher amounts of genistein detoxification and elimination via the feces. Therefore, less genistein remained available to become incorporated into somatic and visceral tissues. While directly analyzing ontogenetic shifts and glucuronidation activity within the digestive tract where beyond the scope of this dissertation, such explanations seem plausible when looking at the percentage changes of genistein found within mean whole-body tissues as compared to the mean fecal matter concentration between the mid- and end-points.

Patterns among treatments seen in the whole-body tissue samples followed genistein concentrations in liver samples. However, unlike the previous Rainbow Trout samples, the overall amount of genistein found within liver tissues was greatly reduced compared to whole-body and fecal samples, accounting for <1% of the total genistein found in these samples for both the NoGen and HiGen treatments at both sampling points. In comparison, another study sought to determine the tissue distribution of genistein by feeding radio-labelled genistein to Rainbow Trout and determining the percentage of radioactivity remaining in the tissues at 48 and 72 h post ingestion (Gontier-Latonnelle et al., 2007). The results indicated that the liver, intestinal fat, and muscles account for 0.5, 0.21, and 0.14% of ingested radioactivity at 48 h and 0.1, 0.09,

and 0.02% at 72 h, respectively; the remaining tissues of gonads, skin, gills, spleen, posterior kidney, and anterior kidney accounted for <0.04% each for both sample times (Gontier-Latonnelle et al., 2007). Similar to reduction of genistein concentrations over time seen in whole-body samples, the reduction in the liver could also be explained via similar ontogenetic changes to the liver. A study looking at the development of livers in 32 to 300 days post-hatch (dph) Rainbow Trout showed the liver to contain hepatocytes with numerous vacuoles, no to low numbers of sinusoids in liver tissue, and no lobule structure by 59 dph (Çinar & Şenol, 2005). By 120 dph, many sinusoids, enlarged central veins, and bile ducts were observed; however, no lobule abnormality was detected until 300 dph, which were reported as uneven in shape (Çinar & Şenol, 2005). Therefore, continued development of the liver would result in increased liver functions associated with increased detoxification processes. Glucuronidation activities were also present in the hepatocytes of liver tissue and were shown to assist in detoxification of genistein in Atlantic Salmon hepatocytes (Olsvik et al., 2017). This same study also indicated that genistein was metabolized by the cytochrome P450 system and may play an important role prior to glucuronidation (Olsvik et al., 2017). Therefore, taken together with the current genistein concentration trends seen in the liver and whole-body samples, continued development of digestive tract and liver, along with their associated detoxification processes, resulted in higher amounts of genistein being excreted via the feces by the end of the current study.

As mentioned previously, there have been a number of CECs that occur in aquaculture effluents due to the production of waste products produced from the aquaculture production process (Dauda et al., 2019). Within intensive culture systems

where artificially produced feeds are necessary, solid wastes consisting of uneaten feed and discharged fecal material are considered the most dangerous waste within the culture system and are to be removed as quickly as possible resulting in varying degrees being released into the environment (Dauda et al., 2019; Ahmad et al., 2021). The current system used within the study mimics that of a flow-through, raceway system albeit not perfectly, which is primarily used for Rainbow Trout culture. The primary problem with such a system is that such solid wastes are remediated within the system at the expense of the environment due to the high flow rates weakly concentrating such effluents making them difficult and expensive to manage and increasing the release to the environment (Dauda et al., 2019). In relation to solid wastes, much attention has been given to the dissolved solids of nitrogen and phosphorus and their associated problems within culture systems and eutrophication in water bodies receiving effluent (Ahmad et al., 2021, 2022) However, other CECs have begun to receive research attention such as antibiotics, fertilizers, anesthetics, and steroid hormones to name a few (Ahmad et al., 2021, 2022). As noted above, genistein concentrations occurring at relevant biological levels make it a potential candidate to consider for CEC research with concentrations seen in sampled fecal material warranting further merit to this claim. While genistein concentrations decreased over time in the whole-body and liver samples, increases were seen in both the fecal and water samples. While genistein leaching into the water from fecal material was not directly determined, it would seem plausible that breakdown of feces prior to removal or left within the system could have resulted in the elevated levels of genistein in the water providing a primary method of contamination into the environment due to the high flow-through rates associated with such culture systems. Likewise, if not handled and

processed properly, the feces themselves as settled solids could still contain un-incorporated and/or excreted concentrations of genistein resulting in further environmental contamination. Such contamination could occur from the use of treated and removed solid waste products for agricultural purposes such as fertilizer, compost, or substrate for biogas productions (Bergheim et al., 2019). Therefore, based on this current research, genistein should be considered a CEC after being found at elevated levels in aquaculture effluent sources from a SBM-based aquaculture diet that mimics current levels used in commercial production.

Level I fugacity modelling

When compared to the empirical data, Level I modelling results found varying levels of model performance at the different treatment levels. As mentioned earlier, Level I models are easily calculated once the necessary compartment data is obtained and provides insight into overall chemical partitioning and estimated concentrations within those compartments (Mackay & Paterson, 1991). Due to the lipophilic of genistein (Gonteir-Latonelle et al., 2007), it would be assumed that a majority of the genistein would be found in the fish compartment of the model. This occurred and was confirmed by comparing calculations of genistein percentages of the empirical data between fish and water (i.e., no data for genistein in air) and similar percentages for fish, water, and air compartments calculated for model estimates. For both empirical and model calculations, >99.99% of the total genistein was found in the fish compartment. Therefore, the Level I models did accurately determine the overall chemical partitioning of genistein within the culture system for both L2W and TGA model sets.

Out of the eight Level I fugacity models performed for both fish and water estimates of genistein, at least one treatment within each model fell within the acceptable PBIAS range indicating that, to some degree, the models did an adequate job of predicting genistein concentrations within these two compartments. This fact is surprising considering the simplicity of calculations and underlying assumptions mentioned in the methods section. These findings support the general premise that at low concentrations of the chemical of interest, fugacity is directly proportional to the concentration, thereby making calculations and estimates much simpler than opposing concentration based models (Yenigun & Sohtorik, 1995; Mackay, 2001). However, caution needs to be taken as no definitive pattern of model acceptability was seen and results for the water comparisons are questionable due to 10 out of the 12 measurements analyzed were below the detection limit of <20 ng/L. For calculation of PBIAS and previous comparisons of the empirical data, the assumed value of 20 ng/L was used for these samples below detection samples. Therefore, while the use of this value should be considered conservative for other calculations, it would affect the model and increase the PBIAS as a decrease in empirical values would increase the difference between overestimated model results. Also, most of the information and comparisons surrounding the Ref treatment, while useful, are less important as this diet was formulated to contain no genistein. The NoGen and HiGen diets provide more useful data and modelling comparisons based on genistein inclusion via SBM and genistein addition. It should be noted that a low genistein concentration was seen in the Ref diet and could be explained by the presence of genistein in other feed ingredients used; however, only SBM and the subsequently produced diets were analyzed for genistein concentrations prior to use in the feeding trial.

For practical use of SBM in aquafeeds, primary attention should be directed to the NoGen treatment which more closely simulates current SBM levels used in culture diets and the HiGen treatment if genistein is added as a supplement to such diets.

Results from Level I models for the NoGen and HiGen treatments indicated several different tendencies of this modelling based on the total inclusion of genistein (*M*). The overall trends showed that no matter which of the *M* amounts were used (i.e., L2W vs. TGA), the model results for the NoGen and HiGen treatments either under- and overestimated, with respect to the empirical data. Such trends seem to suggest that while the model performed well at determining the overall percentage of genistein partitioning between the compartments, it did not have the same performance in determining the actual concentrations within the compartments. Along with this fact, the overall model performance becomes more evident based on PBIAS results. For the empirical fish data, an interesting inversion of model performance occurred between the two genistein data sets. When L2W genistein data was used within the models, the NoGen treatment performed poorly (>84%), while the HiGen treatment performed within the acceptable performance range (-32 to -15%). Conversely, the use of TGA resulted in acceptable performance of the NoGen treatment (6 to 61%) with poor performance of the HiGen treatment models (<-211%). Such differences indicate the importance of determining correct *M* and the overall assumption of chemical conservation within the system. As mentioned earlier, one assumption for Level I fugacity modelling is that *M* is conserved and partitioned within the compartments of the model. Thus, the overall levels used for each data set may have matched a similar pattern as that of the genistein being conserved within the fish. The lower L2W genistein inclusion exhibited a similar conservation trend

as that of the HiGen treatment empirical data but overestimated when larger amounts of genistein were present in the TGA data, while the reverse occurred in the NoGen treatment. While genistein did appear to be conserved within the fish, it is apparent that the total conservation assumption was not met and additional processes (i.e., biodegradation, biotransformation, etc.) affected the overall genistein concentrations within the fish. It appears that such bioprocesses resulted in lower amounts of genistein within fish from the HiGen treatment resulting in decreased model performance as the *M* used in the models increased, while the reverse would be true for the NoGen treatment. Such differences point to the need for careful consideration of the *M* when using Level I models as it affects both the overall calculated fugacity used for partitioning calculations and the amount of genistein available for partitioning. Based on this research and currently used levels of SBM in aquaculture diets, the use of total genistein fed over 56-112-day periods for *M* should be used in similar Level I fugacity models.

While these trends seemed rather distinct for fish, the Level I models for water provided more convoluted results. Only two models showed acceptable performance; the HiGen treatment at the end-point (PBIAS = -18%) and the NoGen treatment at the midpoint (PBIAS = 21%). Such varying results indicate that while Level I models can predict genistein concentrations with the water compartment acceptably at times, there appeared to be no apparent pattern or overall relationship that produced such results. Such findings along with differences seen for comparisons between the fish data and model estimates indicate that Level I model limitations exist. Some of these limitations are most likely due to the base assumptions necessary for Level I fugacity models. One such assumption is that when equilibrium exists within each compartment a common

fugacity applies to the whole system and is used to determine estimated chemical concentrations within each compartment (Mackay & Paterson, 1991; Mackay, 2001). However, this assumption is usually in error as each compartment can have differing fugacity with a higher fugacity occurring in compartments receiving direct discharges of the chemical and lower fugacities in those having relatively rapid loss mechanisms (Mackay & Paterson 1991). Likewise, it is assumed that all compartments reach equilibrium in a relatively short period of time and that no additional processes occur resulting in the addition or loss of the chemical from the system. Such assumptions were difficult to maintain given the nature of this experiment (i.e., emulating a feeding trial) and the complexity associated with additional processes occurring within the system and fish that affect potential genistein concentrations in the model compartments. Therefore, the use of Level I models to estimate genistein in the various model compartments should be used with caution and only as a preliminary means of determining chemical behavior within an aquaculture system. However, these findings merit the potential use of such models if further research and revision occurs to address current model assumptions that were not met, especially given the relative ease of calculations needed for Level I calculations making them accessible to a wide array of users.

Level II fugacity modelling

Fugacity modelling consists of four levels of sophistication that include attributes of Level I being listed above. Level II models increase in complexity and are useful for studying the potential processes that control environmental fate of chemicals along with determining chemical partitioning and estimated concentrations within differing compartments (Yenigun & Sohtorik, 1995; Mackay, 2001). Similar to the chemical

partitioning percentages seen in Level I model estimates, Level II model estimates indicated similar chemical partitioning between the fish and water compartments with >98.98% and <1.02%, respectively. From these results, both Level I and II fugacity models performed adequately in determining the overall partitioning of genistein within the currently studied aquaculture microcosms.

The increased complexity of Level II models occurs from the inclusion of the chemical flow into and out of the purposed compartments within each microcosm via input, emission, advection, and degradation rates. Compared to Level I calculations, the amount of chemical available is no longer the total amount (i.e., mols) available to the system but rather a rate (i.e., mol/hr), which is necessary to address the effects of advection and degradation rates. It is better to view Level II models as providing a "snap-shot" of genistein concentrations within the compartments based on the rates and the assumption that emission rates equal the inflow. Therefore, it is not surprising that Level II models highly underestimated the amount of genistein in each compartment, except water in the HiGen treatment at the mid-point (44 to 63%) for both L2W and TGA data sets. Such underestimation is likely the result of the failure to meet several of the assumptions necessary for Level II models. The assumption of all compartments reaching steady-state equilibrium may not have occurred as the empirical data showed statistically significant changes of genistein concentrations within the fish and water compartments from the mid- to end-points. This supports the case that such conditions rarely occur in most scenarios as indicated in other sources (Jørgensen & Bendoricchio, 2001; Mackay, 2001). In another study of fugacity modelling, ethiprole was added as a direct solution stock to the microcosms via the water inflow and did not reach a steady-state until 14

days after initiation (Hu et al., 2017). While the current study allowed a similar 14-day period (i.e., L2W data) and measured rates over the entire study period (i.e., TGA data), it is possible that genistein did not reach equilibrium in all compartments due to the chemical being added to the system via food and not direct aqueous stocks. This mode of introduction likely resulted in a longer period necessary to reach equilibrium. Likewise, it was assumed that all *V* and *Z* values remained constant with time, which was not the case as the current study also sought to mimic a growth study resulting in fluctuations to *V* and subsequently *Z* values. While not greatly different, the slight improvement of model results using the L2W data in place of the TGA data (Figure 4-4) would support this claim as it utilized genistein inflow rates that were closer to the input *V* and *Z* values for the model based on the sampling periods. Along with these changing values, the ontogenetic shifts occurring in the fish as mentioned in the Level I model discussion above may have shifted the overall equilibrium pattern between fish and water compartments resulting in a more non-steady state situation.

The overall underestimation of Level II models could also be explained by the inclusion of the degradation reactions (i.e., D_R) occurring in all 3 compartments. Such reactions are considered chemical loss processes that alter the chemical nature of the solute and include such processes as biodegradation, photolysis, oxidation, and hydrolysis (Mackay & Paterson, 1991; Mackay, 2001; Chen et al., 2021). In essence, the chemical is being “lost” from the compartments due to its alteration of the parent chemical into different chemical form. For Level II models of genistein, the literature indicated several such reactions (Table 4-2), which were also necessary for calculation of the systems fugacity needed for determining model estimates (Mackay & Paterson, 1991;

Mackay, 2001). However, these reactions resulted in a chemical loss within each compartment analyzed. This behavior of the model, along with the assumption of equal inflow to outflow, results in greater amounts of the chemical being lost from the compartments and calculated as outflow. These results stand in contrast to those seen and mentioned above for Level I models where satisfactory modelling results occurred, albeit dependent upon the overall *M*. Therefore, genistein remained intact (i.e., no biodegradation or biotransformation) within the compartments and was conserved in Level I estimates versus a flow rate through the system and compartments resulting in outflow of the chemical. Likewise, it is likely that, while reported in the literature, the reaction rates used may not be directly applicable to the current microcosm system and have resulted in increased variability of estimates. For example, the oral half-life of genistein from Rainbow Trout was analyzed using adult Rainbow Trout at 16°C, whereas this study utilized larval to juvenile Rainbow Trout cultured at a lower temperature. No half-life for genistein in larval to juvenile trout could be found in the literature. Other differences also occurred for the remaining two degradation rates, thus placing additional error inherently into the model. However, such an approach provides more plausible grounds for defense of the results versus using estimated or simply guessed rates to produce adequate model estimates. Also, as mentioned earlier for Level I limitations, the assumption of a common fugacity was most likely unmet by the Level II models.

Conclusion

This current research has demonstrated that genistein introduced into an aquaculture system via diets can be found within both the cultured fish and water. The presence of genistein was also found within the two primary aquaculture effluent

constituents of from culture production, culture water and solid wastes from fed fish. Genistein measured within the culture water of the microcosms occurred at similar or higher levels as seen in other surface waters contaminated from various effluent sources and concentrations shown to produce negative biological impacts on exposed species. While research on potential effluent contaminants from aquaculture production has focused on a various chemicals and nutrients used for production and treatment of fish this research suggests the potential for negative impacts of naturally derived phytoestrogens found in aquafeeds using SBM at commonly used levels (i.e., 20% inclusion rates). In addition, whole-body samples contained genistein in the µg/g scale, which could provide a potential exposure pathway to human consumers. However, due to the lipophilic nature of genistein and previous research on genistein in fish fillets, most of the genistein found in cultured fish should be found in the fatty viscera and organs versus the muscle tissue and thus, not pose a significant health risk. However, more research is warranted to determine genistein partitioning in products produced via aquaculture using SBM-based diets to determine potential dietary genistein levels within such products and their associated risk to the consumer as both SBM and aquaculture continue to increase globally. Along with these research suggestions, further research is needed to determine the potential pathways and removal mechanisms occurring within the digestive tract of cultured fish. The changes of genistein concentrations seen in whole-body, liver, fecal, and water samples seem to suggest that ontogenetic changes occurring in the digestive tract and liver resulted in increased excretion of genistein from the fish and into the culture water. While some research has been performed to assess genistein effects, the exact mechanism and corresponding genistein conjugates remain relatively unstudied in

fish species and seem follow normal biotransformation pathways similar to other lipophilic compounds. The current research did not attempt to determine the different types and levels of genistein conjugates as the primary focus was to provide an empirical data set for model comparisons. However, determination of conjugates and their levels over the development of digestive ontogeny of larval fish could help to elucidate the underlying mechanisms and should be pursued.

The Level I and II fugacity models developed for this study provided several interesting observations when compared against the empirical data set and model estimates. While generally considered simple and less used than the higher-level fugacity models, Level I models provided acceptable estimates for the NoGen and HiGen treatments but was dependent upon the amount of dietary genistein to determine fugacity and availability for apportioning. Such findings are surprising given the simple nature, assumptions to be met, and relative lack of use of Level I fugacity models seen in the literature. Therefore, further use of such models in highly controlled aquaculture settings is merited as it could provide a convenient but acceptable way of estimating potential genistein exposure to cultured fish and concentrations from culture tanks/pond effluents. However, more research and modelling verification is needed on both genistein and other isoflavones to determine the efficacy and repeatability of using Level I models. In contrast to Level I fugacity models, Level II models highly underestimated genistein concentrations in the studied compartments. It is probable that most of the assumptions necessary for such modelling where unmet within the actual culture microcosms and that the increased complexity needed (i.e., inflow, outflow, and degradation rates) added error within the model resulting in poor model performance. In similar fashion, more

sophisticated models such as Level III and IV fugacity models would meet more of the assumptions needed for the current research, could be used to provide more refined estimates, and have been shown fairly accurate in the study of other chemicals (Hu et al., 2017; Chen et al., 2021). However, such model analyses require additional data that needs to be studied and/or developed empirically prior to modelling.

Table 4-1. Model parameters and description of volumes (V), fugacity capacity (Z), degrading reactions (k), and associated reaction rates (D) values needed for various compartments of fish ($_F$), water ($_W$), and air ($_A$) used in the proposed fugacity model.

Compartment	Formula	Description
Volumes (V)		
Fish	V_F	Total fish volume per microcosm
Water	V_W	Total volume of water per microcosm
Air	V_A	Total volume of air laboratory
Fugacity Capacities (Z) [a]		
Fish	$Z_F = L_F K_{OW} \rho_F / H$	L_F is mean fish lipid fraction per microcosm K_{OW} [b] is listed in Table 4-2 ρ_F is the density of fish per microcosm H [b] is listed in Table 4-2
Water	$Z_W = 1/H$	H is listed in Table 4-2
Air	$Z_A = 1/RT$	R is gas constant (8.314 Pa m^3/mol K) T is temperature (°K)
Degrading Reactions (k)		
Fish	$k_{Fo} = 0.693/\tau_{½}(oral)$	$\tau_{½}$ [b]$(oral)$ is listed in Table 4-2
Water	$k_W = 0.034\ h^{-1}$	Kelly et al. (2014) in Table 4-2
Air	$k_A = 0.693/\tau_{½}(photolysis)$	$\tau_{½}(photolysis)$ is listed in Table 4-2
Reaction Rates (D)		
Fish	$D_F = V_F Z_F k_F$	
Water [a]	$D_I = V_W Z_W k_W$	
Air [a]	$D_W = V_A Z_A k_A$	

[a] Values and formulas found in Mackay (2001).

[b] K_{OW} is the octanol-water partitioning coefficient, H is Henry's Law Constant, and $\tau_{½}$ is a half-life.

Table 4-2. Chemical properties of genistein used in calculations for the Level I and II fugacity models in this study.

Property	Value	Unit	Reference
Henry's Law constant [a]	5.10×10^{-17}	atm m^3/mol	NCBI (2016)
	5.20×10^{-12}	Pa m^3/mol	NCBI (2016)
Solubility	3.32×10^{-6} at 286°K [b]	mol/L	Wu et al. (2010)
	3.32×10^{-9} at 286°K [b]	mol/m^3	Wu et al. (2010)
	5.30×10^{-6} at 298°K	mol/L	Wu et al. (2010)
	5.30×10^{-9} at 298°K	mol/m^3	Wu et al. (2010)
Vapor pressure [a]	5.20×10^{-12} at 298°K	mm Hg	NCBI (2016)
	6.90×10^{-10} at 298°K	Pa	NCBI (2016)
Molar mass	270.24	g/mol	Murphy et al. (2002)
Octanol-water partitioning coefficient (K_{OW})	1096.5	N/A	Rothwell et al. (2005)
Half-life ($\tau_{½}$ intravascular; Rainbow Trout)	2.025 at 16°C	h	Gontier-Latonnelle et al. (2007)
Half-life ($\tau_{½}$ oral; Rainbow Trout)	13.075 at 16°C	h	Gontier-Latonnelle et al. (2007)
Half-life ($\tau_{½}$ photolysis)	25 at pH 8	h	Felcyn et al. (2012)
	5.30 at pH 10	h	Felcyn et al. (2012)
Degradation rate [c] (k; water)	0.092-0.231 at 100 mg/L	h^{-1}	Kelly et al. (2014)
	0.080 at 50 mg/L	h^{-1}	Kelly et al. (2014)
	0.071 at 10 mg/L	h^{-1}	Kelly et al. (2014)
	0.036 at 1 mg/L	h^{-1}	Kelly et al. (2014)
	0.013-0.034 at 0.5 mg/L	h^{-1}	Kelly et al. (2014)

[a] Estimated value.
[b] Solubility of genistein in water. Estimated from data set in Wu et al. (2010) to obtain solubility at test water temperature (i.e., 13°C = 286°K).
[c] First-order degradation (k) rates experimentally determined.

Table 4-3. Level I fugacity calculations used to determine genistein concentration estimates and chemical rates in the proposed fugacity model. Form adapted from original in Mackay (2001).

Description (Unit)	**Symbol/Formula**	**Compartments**		
Total Chemical Amount (mol)	M			
Active input rate		*Fish*	*Water*	*Air*
Volume (m^3)	V			
Fugacity capacity (mol/m^3 Pa)	Z			
Volume × Fugacity capacity	VZ			
Fugacity	$f = M/\Sigma VZ$			
Concentration calculations		*Fish*	*Water*	*Air*
Concentration (mol/m^3)	$C = Zf$			
Moles (mol)	$m = CV$			
C_G i.e., C_W (g/m^3)	$C_G = C$*molar mass			
Density (kg/m^3)	ρ			
C_U (µg/g)	$C_U = C_G*(1{,}000/\rho)$			

Table 4-4. Level II fugacity calculations used to determine genistein concentration estimates and chemical rates in the fugacity model. Form adapted from original in Mackay (2001).

Description (Unit)	**Symbol/Formula**	**Compartments**		
Active input rate		*Fish*	*Water*	*Air*
Chemical inflow rate (mol/h)	I			
Total input rate	$E = I$			
D value calculations		*Fish*	*Water*	*Air*
Volume (m^3)	V			
Fugacity capacity	Z			
Volume × Fugacity capacity	VZ			
Reaction half-life (h)	$\tau_{1/2}$			
Rate constant	$k = 0.693/\tau_{1/2}$			
D reaction	$D_R = VZk$			
D advection	$D_A = GZ$			
Total D per compartment	$D_T = D_R + D_A$			
Total D value	$Total\ D = \Sigma D_T$			
Fugacity	$f = I/\Sigma D$			
Concentration calculations		*Fish*	*Water*	*Air*
Concentration (mol/m^3)	$C = Zf$			
Moles (mol)	$m = CV$			
C_G i.e., C_W (g/m^3)	$C_G = C_W$			
Density (kg/m^3)	ρ			
C_U (µg/g)	$C_U = C_G(1{,}000/\rho)$			

Table 4-5. Genistein levels in whole-body, fecal and liver samples taken from Rainbow Trout fed a reference (Ref) diet or diets with genistein at 30.6 (NoGen), and 2,010 µg/g (HiGen) and in culture water collected from microcosms. Means ± standard deviations listed with range shown inside parenthesis (n = 6). Values not significantly different (P ≥ 0.05) have the same letter for a given dependent variable. Asterisk (*) indicates statistical difference between mid- and end-points.

	Whole-body (µg/g)		**Fecal (µg/g)**		**Liver (µg/g)**		**Water (ng/L)**	
DOE	**56***	**112***	**63***	**119***	**56***	**112***	**56***	**112***
Ref	2.92 ± 3.14 x (1.05-9.10)	2.25 ± 1.53 x (0.825-4.33)	1.16 ± 0.04 x (1.10-1.20)	1.72 ± 1.23 x (0.75-4.05)	0.129 ± 0.039 x (0.077-0.185)	0.079 ± 0.038 x (0.034-0.149)	25.5 ± 13.4 y (<20[a]-52.9)	26.3 ± 15.3 y (<20[a]-57.5)
NoGen	102 ± 28.0 y (55.0-138)	56.8 ± 39.8 y (30.6-136)	13.9 ± 21.0 y (2.85-56.6)	57.5 ± 63.0 y (11.4-181)	0.926 ± 0.479 y (0.548-1.81)	0.632 ± 0.217 y (0.365-0.903)	779 ± 680 z (2.85-56.6)	2,488 ± 620 z (1,690-3,010)
HiGen	830 ± 523 z (271-1,681)	459 ± 273 z (269-998)	172 ± 89.4 z (75.4-336)	1,498 ± 528 z (518-2,036)	3.71[b] ± 1.45 z (2.47-5.61)	2.36 ± 0.823 z (0.778-3.17)	1,951 ± 1,438 z (66.1-3,770)	5,195 ± 4,105 z (1,610-12,100)

[a] Assumed 20 ng/L for descriptive statistics and statistical analyses in 5 replicates with measured values of <20 ng/L.
[b] Samples for 1 replicate lost by USGS, Columbia Environmental Research Center during processing leaving n = 5.

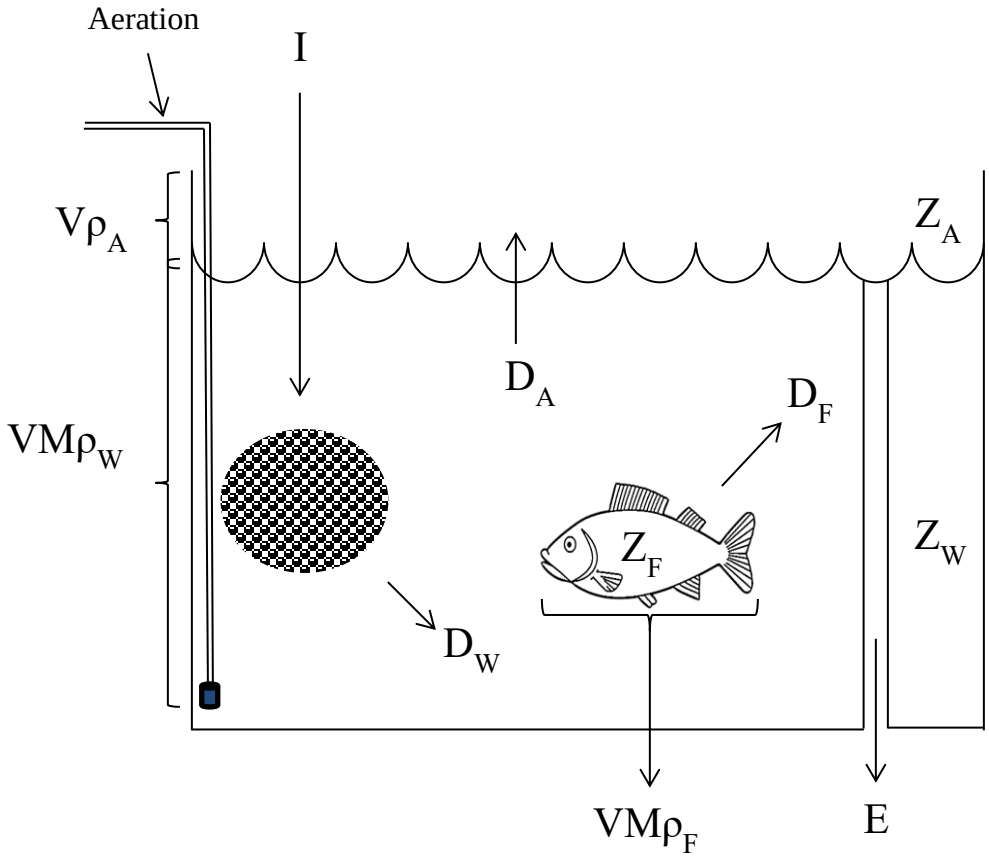

Figure 4-1. Schematic diagram of aquatic microcosms and associated parameters necessary for Level I and II fugacity model calculations of genistein. Parameters include inflow (I), volume (V), mass (M), density (ρ), fugacity capacity (Z), reaction rate (D), and emission (E) for model compartments of water ($_W$), fish ($_F$), and air ($_A$).

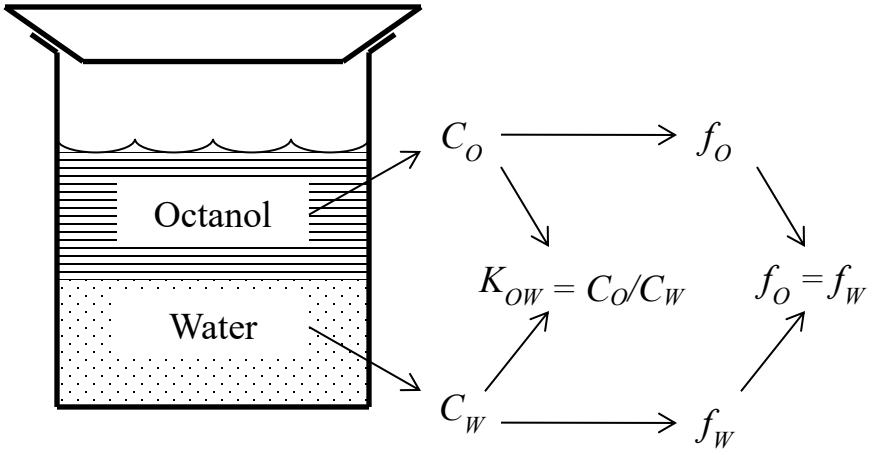

Figure 4-2. A schematic depicting the equilibrium criteria approach using both concentration (*C*) and fugacity (*f*) of a chemical in the phases of octanol ($_O$) and water ($_W$) with the corresponding octanol-water partitioning coefficient (K_{OW}). Figure modified from Mackay (2001).

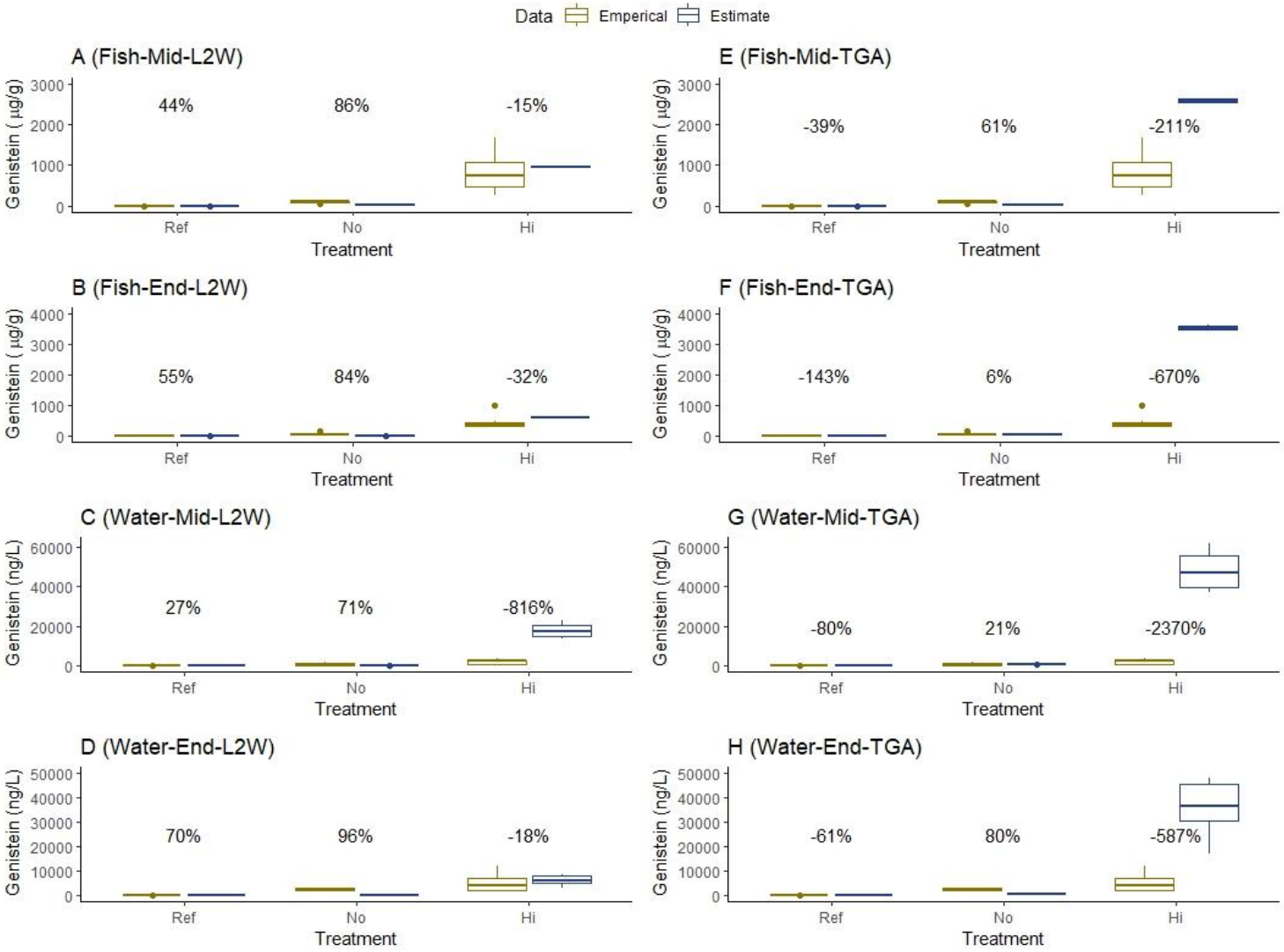

Figure 4-3. Empirically measured and Level I fugacity model estimates of genistein concentrations in whole-body fish samples (Fish) and culture water (Water) at the mid- (Mid, 56 DOE) and end-points (End, 112 DOE). Models used fed genistein amounts during the two-week period (L2W) prior to sampling points and overall totals (TGA). Listed percentages are associated PBIAS estimates for each pairing of empirical data and associated model estimates.

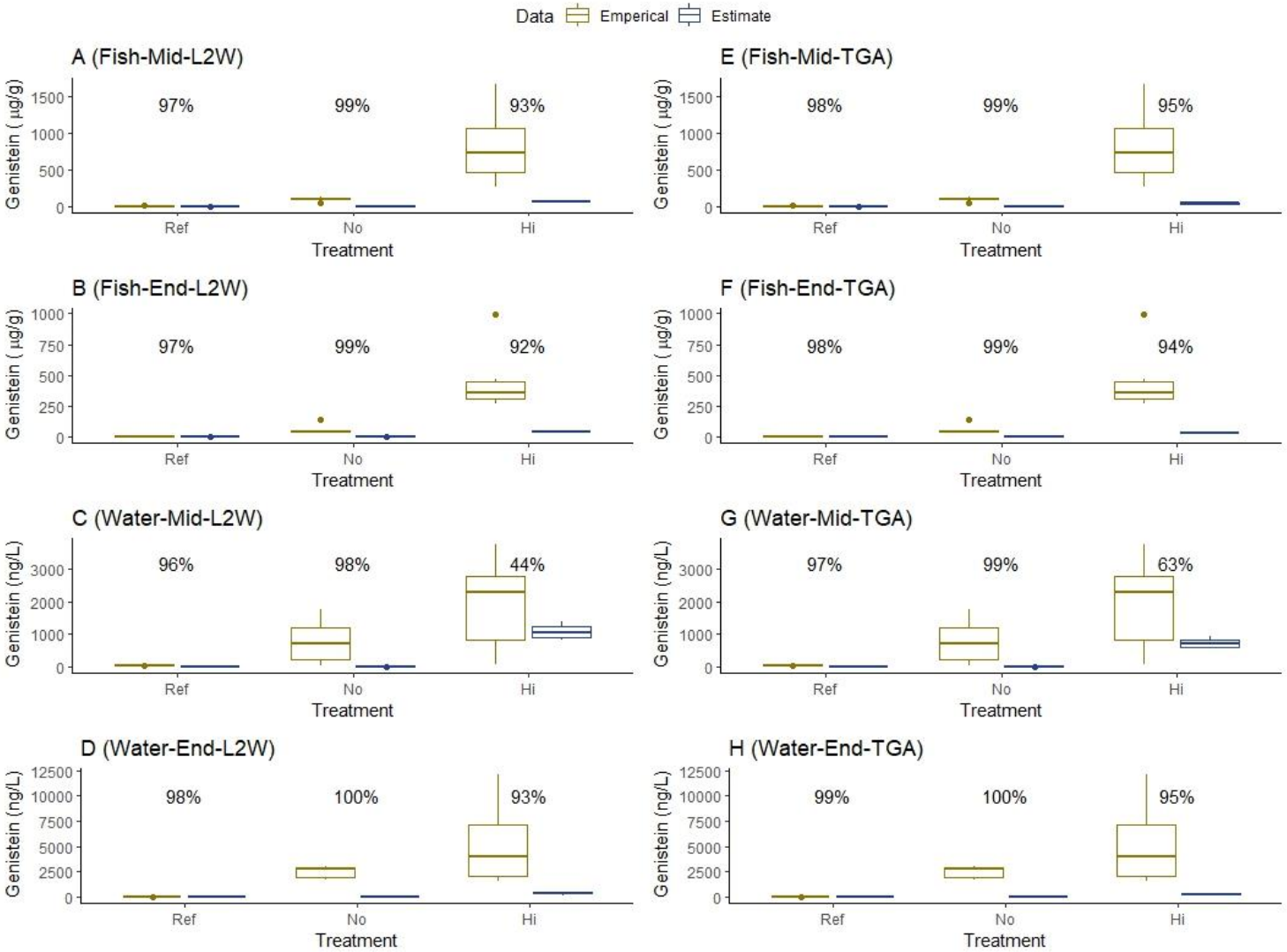

Figure 4-4. Empirically measured and Level II fugacity model estimates of genistein concentrations in whole-body fish samples (Fish) and culture water (Water) at the mid- (Mid, 56 DOE) and end-points (End, 112 DOE). Models used estimated fed genistein rates during the two-week period (L2W) prior to and up to (TGA) each sampling period. Listed percentages are associated PBIAS estimates for each pairing of empirical data and associated model estimates.

APPENDICES

Appendix A. Analytical information on the sample processing and analysis of the soybean meals and experimental diets used as performed by the Unites States Geological Survey, Kansas Water Science Center, Organic Geochemistry Research Laboratory (OGRL) to determine phytoestrogen levels.

Analytical Method Information

Phytoestrogen Solid Method: LCHS_PE

Prepared: 02/22/2021 by OGRL staff

The phytoestrogen method for the analysis of biochanin A, coumestrol, daidzein, equol, formononetin, and genistein in solid matrices was developed at the Kansas Water Science Center, Organic Geochemistry Research Laboratory (OGRL) and was adapted from a 37-compound solid-phase extraction liquid chromatography/tandem mass spectrometry (UPLC/MS/MS) method (Yost et al., 2014). The method uses accelerated solvent extraction (ASE) for extraction and UPLC/MS/MS for analysis. Solid samples were shipped overnight on ice to OGRL and stored at -20°C until extraction.

Solid samples were prepared for extraction by thoroughly homogenizing 1 g of sample and 0.5 g of hydromatrix (Agilent, Santa Clara, California) with a mortar and pestle. A 1:10 dilution was prepared by adding 0.5 g of the first mixture to 4.5 g of hydromatrix and homogenizing with a mortar and pestle. Serial dilutions at 1:100, 1:1,000, and 1:10,000 were prepared similarly. A 1.5 g aliquot of each sample mixture was packed into a 5 mL stainless steel cell preloaded with a glass fiber filter and baked Ottawa sand and then spiked with surrogate internal standard mix; standard addition samples were spiked with standard mix. Remaining void space in each cell was packed with additional Ottawa sand and the cell was capped and loaded onto the Dionex ASE 350 (ThermoFisher, Waltham, Massachusetts). The samples were extracted using a 50/50

(v/v) solution of 0.3 M citric acid buffer (pH 5.9) and methanol at 50°C with three 2-min static cycles, a 120-second purge cycle, and 150% rinse volume resulting in approximately 15 mL of extract. Extracts were evaporated at 40°C to 7.5 mL and brought back to 15 mL with Type I water; 1 mL was vialed in Waters (Milford, Massachusetts) max recovery vial and stored at -20°C until analysis. Standard curve, check standards, and laboratory blank samples were prepared in a 2:1 mix of Ottawa sand and hydromatrix. A duplicate sample and duplicate standard addition sample were prepared for each sample at each dilution analyzed and a laboratory blank was run after every sample set.

Solid samples were analyzed for the 6 phytoestrogens by UPLC/MS/MS with electrospray ionization (ESI) and multiple reaction monitoring (MRM) with Daidzein-D_3 and Genistein-D_4 as surrogate internal standards. A laboratory blank and check standard were analyzed at least every tenth sample. All standard curve samples, laboratory blanks, and check standards were treated the same as the environmental solid samples. Standard curve samples were prepared at concentrations of 0.5, 1, 2, 5, 10, 50, 100, and 500 ppb (µg/kg), check standards were prepared at 2 and 50 ppb (µg/kg), the standard addition samples were spiked at 50 ppb (µg/kg), and the surrogate internal standards were spiked at 100 ppb (µg/kg) for Daidzein-D_3 and Genistein-D_4.

The samples were analyzed using a Waters Acquity H-class Bio UPLC with a Sciex (Framingham, Massachusetts) API 5500 Triple Quadrupole Mass Spectrometer. For separation a vanguard pre-column and Waters Acquity BEH 1.7 µm, 50x2.1 mm UPLC analytical column were used with a (A) water, (B) methanol, and a post-column infusion of 10 mM ammonium hydroxide mobile phase. MS positive-ion mode was used

with electrospray ionization and two multiple reaction monitoring (MRM) transitions were selected for each analyte and identification was based on the retention time and the ratio of the two MRM transitions being within +/-25%. A linear 1/x weighting was applied to the calibration curve and detected compounds were quantitated using standard addition calculation and with the percent moisture accounted for. To meet quality control specifications environmental samples and duplicates did not exceed a 20% difference, the target matrix spike recovery range was within 80-120% for analytes with matching surrogate internal standards, and the check standard accuracy was within 80-120%. The reporting limit was 1 ppb (µg/kg).

Appendix B. Fish species with size/life stage, phytoestrogens used, reported phytoestrogen levels, fish responses and relevant results from studies analyzing phytoestrogen effects on cultured finfish.

Fish Species (Size/Life stage)	Phyto-estrogen(s)	Phytoestrogen Level	Response	Comments	Reference
Salmonids					
Rainbow Trout (mean weight = 500 g)	Genistein Daidzein Coumestrol Equol Formononetin Biochanin A	N/A	1,000-2,000X[a] less active than estradiol.	*In vitro* technique. Similar estrogenic activity to androgens and progestogens.	Pelissero et al. (1993)
Rainbow Trout (mean weight = 83 g)	Genistein Daidzein	2.9, 20.0, 11.1, 30.4, 105, 2.9, and 70.2 mg genistein/kg diet 5.4, 19.8, 19.8, 19.7, 61.0, 5.2, and 56.5 mg daidzein/kg diet	2.1-22.6 mg genistein/kg in bile. 2.5-10.6 mg daidzein/kg in bile. Higher VTG[b] at study end. No VTG changes between treatments.	Dietary exposure via soy flour and SPC[c]. Listed genistein and daidzein correspond to seven diets	Kaushik et al. (1995)
Rainbow Trout (initial weight = 106.0 g)	Genistein Daidzein	1,084, 4,042, and 4,525 mg genistein/kg diet 594, 1,476, and 1,362 mg daidzein/kg diet	0.51-1.03 mg daidzein/kg in bile. 0.21-0.56 mg genistein/kg in bile. Reduced intake and growth of soy protein concentrate diets.	Dietary exposure via SPC (5,903 mg genistein/kg; 1,990 mg daidzein/kg diet). Listed genistein and daidzein in three diets.	Mambrini et al. (1999)
Rainbow Trout (initial weight = 40 g)	Genistein	0, 500, 1,000 mg genistein/kg diet	Growth not affected. Some effects on reproductive performance.	Dietary exposure. See manuscript for details on responses.	Bennetau-Pelissero et al. (2001)
Rainbow Trout (weight = 500-1,000g)	Genistein	N/A	30-200X less active than 17β-E2[d]. 2.1×10^{-7} equilibrium inhibitory concentration. 3.6 relative binding affinity.	*In vitro* technique.	Tollefsen et al. (2002)
Rainbow Trout (adult females)	Genistein Coumestrol	N/A	Genistein-1.22 relative affinity, 570 nM DC_{50}[e] value.	*In vitro* technique.	Latonnelle et al. (2002)

	Formononetin Equol Daidzein Biochanin A		Coumestrol-1.75 relative affinity, 400 nM DC_{50} value. Formononetin-2.69 relative affinity, 260 nM DC_{50} value. Equol-0.13 relative affinity, 5,300 nM DC_{50} value. Daidzein-0.077 relative affinity, 9,050 nM DC_{50} value. Biochanin A-<0.007 relative affinity, >100,000nM DC_{50} value. 10-100X lower affinities than estradiol.		
Rainbow Trout (mean weight = 350 g)	Genistein Daidzein Formononetin Biochanin A Equol	0.0, 0.2, 2.0, and 20 mg genistein/kg diet	No VTG differences between dietary treatments. Genistein 20-100X less active than E2. Formononetin 60-300X less active than E2.	Dietary exposure with genistein only. *In vitro* technique with remaining phytoestrogens.	Latonnelle, LeMann, et al. (2002)
Rainbow Trout (fry; mean weight = 4.5 g)	Genistein	0, 500, 1,000, or 3,000 mg genistein/kg diet	No differences in growth, proximate composition, color, and genistein levels. TBARS[f] higher in all genistein diets and 500 mg/kg at six and 12 months. Positive correlation between dietary and tissue genistein levels.	Dietary exposure.	D'Souza et al. (2005)
Rainbow Trout (total length = 27-33cm)	Genistein Daidzein Glycitein	1 μM genistein 1 μM daidzein 1 μM glycitein 1:0.8:0.2 μM mixture	Increased genistein led to greater inhibition of [^{3}H]E2[g] metabolized. No differences from other phytoestrogens/mixture.	*In vitro* technique.	Ng et al. (2006)
Rainbow Trout (adult, females; mean weight = 1 kg)	Genistein	4 mg/kg body weight (IV) 4 mg/kg body weight* (OR)	Pharmcokinetics - C_{max}=0.012 ng/mL; T_{max}=11.66 h; $T_{1/2IV}$=2.025 h; $T_{1/2OR}$=13.075 h. Bioavailability = 6-13%.	Intravascular injection (IV) and/or oral ingestion (OR; *200 mg genistein/kg).	Gontier-Latonnelle et al. (2007)
Rainbow Trout (initial mean	Genistein Daidzein Glycitein	500 mg mixture/kg (523.14 mg isoflavones/kg; 43.15%	No differences in growth, condition, cortisol, Insulin Growth Hormone I, or histology of various tissues.	Dietary exposure. Diets from mixture of 46.64% genistein:	Pastore et al. (2018)

weight = 176.7 ± 0.7)		genistein; 46.60% daidzein; 10.25 % glycitein). 1,000mg mixture/kg (1,466.60 mg isoflavones/kg; 42.28% genistein; 47.31% daidzein; 10.41% glycitein).	Significant correlation between E2 and VTG production via densitometry.	46.65% daidzein: 6.71% glycitein.	
Rainbow Trout (initial weight = 144.2 ± 16.1 g)	Genistein	0 and 3,000 mg genistein/kg diet	No differences in growth, mortality, and gut morphology. Lower apparent digestibility coefficients of dry matter, crude lipid, and gross energy for genistein diet.	Dietary exposure.	Torno et al. (2018)
Rainbow Trout (initial weight = 81.6 ± 0.5 g)	Genistein	0 and 3,000 mg genistein/kg diet	No differences in growth, condition, hematocrit level, fatty acid composition and most whole-body composition. Higher crude ash level for fish fed genistein diet without fish oil. Lower feed intake for genistein diets. Lower food conversion ratio for genistein diet without fish oil. Higher protein efficiency ratio and protein productive value for genistein diet without fish oil.	Dietary exposure. Genistein level same for two diets with and without fish oil. These were compared against complimentary control diet with no genistein.	Torno et al. (2019)
Rainbow Trout (14 dph[h], mean weight = 0.123 g)	Soybean isoflavone extract	0, 0.10, 0.20 g soybean isoflavone extract/L	Female-skewed sex ratio of 0.20 g/L treatment vs. control. No differences in growth or survival.	Immersion treatment. Isoflavone type and level not determined.	Turan & Yigitarslan (2019)
Atlantic Salmon (total length = 15-20 cm)	Genistein Daidzein Glycitein	1 μM genistein 1 μM daidzein 1 μM glycitein 1:0.8:0.2 μM mixture	Increased genistein led to greater inhibition of [^{3}H]E2 metabolized. No differences from other phytoestrogens/mixture.	*In vitro* technique.	Ng et al. (2006)

Atlantic Salmon (weight = 500-1,000 g)	Genistein	N/A	30-200X less active than 17β-E2. 3.9×10^{-7} equilibrium inhibitory concentration. 0.48 relative binding affinity.	*In vitro* technique.	Tollefsen et al. (2002)
Atlantic Salmon (fry; initial mean weight = 0.17 ± 0.01 g)	Isoflavones	0 (- and + controls) and 1.5 g isoflavones/kg diet	No differences in survival or condition. Lower final body weight. Significant negative impacts on digestive enzyme activities. No differences in intestinal histology. Lower vacuole accumulation and hepatocyte diameter in livers.	Dietary exposure. Isoflavone types and levels not reported.	Gu et al. (2015)
Lake Trout (total length = 30-40 cm)	Genistein Daidzein Glycitein	1 μM genistein 1 μM daidzein 1 μM glycitein 1:0.8:0.2 μM mixture	Increased genistein led to greater inhibition of [^{3}H]E2 metabolized. No differences from other phytoestrogens/mixture.	*In vitro* technique.	Ng et al. (2006)
Channel Catfish					
Channel Catfish (mean weights; male = 2.5 kg, female = 3.2 kg)	Genistein	10^{-9} to 10^{-2} molar-based solutions	Reduced spermatozoa motility time, motility rank, and ATP^{i} content. Reduced fertilization rates. Positive relationship between ATP content and percent fertilized.	Waterborne exposure.	Green & Kelly (2008)
Channel Catfish (fry)	Genistein	2, 4, or 8 mg genistein/g diet	Phenotypic sex dependent on dietary genistein levels. Intersex occurred in 4 and 8 mg diets. Paradoxical sex reversal.	Dietary exposure.	Green & Kelly (2009)
Japanese Medaka					
Japanese Medaka (mean weight = 0.26 g)	Genistein	75, 750, or 30,000 ng genistein/fish	Increased *ex vivo* E2 release in females. Decreased *ex vivo* and circulating testosterone in male fish.	Exposure via injections.	Zhang et al. (2002)
Japanese Medaka (early life stage)	Genistein Equol	1, 10, 100, and 1,000 μg genistein/L, 0.4 and 0.8 μg equol/L	Negative effects on male and female reproductive processes. Intersex in male fish.	Waterborne exposure. See manuscript for details on responses.	Kiparissis et al. (2003)

Japanese Medaka (breeding adults)	Genistein Daidzein	<0.8-58.5 µg genistein/ g diet <0.8-37.3 µg daidzein/ g diet	No differences in growth, condition, or reproductive metrics. Higher hepatic VTG in males fed commercial diet.	Dietary exposure. Six diets analyzed for genistein and daidzein (see Table 2).	Inudo et al. (2004)
Sturgeon					
Siberian Sturgeon (mean weight = 20 g)	Formononetin Biochanin A Daidzein Genistein Equol Coumestrol	0.5 mg formononetin or biochanin A/g weight, 0.2 mg daidzein or genistein/g weight, 0.05 mg equol or coumestrol /g weight	Induced VTG synthesis (except formononetin).	Exposure via injections.	Pelissero et al. (1991)
Siberian Sturgeon (adults)	Genistein Coumestrol Formononetin Equol Daidzein Biochanin A	N/A	Genistein-2.3 relative affinity, 220 nM DC_{50} value. Coumestrol-3.5 relative affinity, 150 nM DC_{50} value. Formononetin-0.5 relative affinity, 1,090 nM DC_{50} value. Equol-0.06 relative affinity, 8,300 nM DC_{50} value. Daidzein-0.006 relative affinity, 83,000 nM DC_{50} value. Biochanin A-<0.005 relative affinity, >100,000 nM DC_{50} value. 10-600X lower affinities than estradiol.	*In vitro* technique.	Latonnelle, Fostier, et al., (2002)
Siberian Sturgeon (18 months old)	Genistein Daidzein Formononetin Biochanin A Equol	0.0, 0.2, 2.0, and 20 mg genistein/kg diet	Increased VTG from 20 mg/kg diet. Genistein-1/900 relative potency. Daidzein-1/700 relative potency. Formononetin-1/500 relative potency. Biochanin A-1/10,000 relative potency. Equol-1/400 relative potency.	Dietary exposure with genistein only. *In vitro* technique with remaining phytoestrogens.	Latonnelle, LeMann, et al. (2002)
Siberian Sturgeon (immature; mean weight = 900 g)	Genistein	4 mg/kg body weight (IV)	Pharmacokinetics - C_{max}=0.27 ng/mL; T_{max}=10.7 h; $T_{1/2IV}$=12.35 h; $T_{1/2OR}$=12.68 h.	Intravascular injection (IV) and/or oral	Gontier-Latonnelle et al. (2007)

		1 mg/kg body weight* (OR)	Bioavailability = 18.9%. Bioaccumulation was low.	ingestion (OR; *200 mg genistein/kg).	
Beluga Sturgeon (5-year-old; mean weight = 13.25 ± 0.3 kg; total length 140.3 ± 1.2 cm)	Genistein	0, 0.2, 0.4, 0.6, and 1.8 g/kg diet	No differences in growth, feed performance, condition, oocyte diameter, sex steroids, or alkaline phosphatase among treatments. Calcium and phosphorus significantly higher in 1.6 g/kg treatment compared to control. Plasma cholesterol significantly higher in 0.8 and 1.6 g/kg compared to control.	Dietary exposure.	Jourdehi et al. (2014)
Russian Sturgeon (100 dph)	Genistein Daidzein Coumestrol	500 mg genistein/kg 10,000 mg daidzein/kg 10 mg coumestrol/kg	Expression patterns of genes *erα*, *vtg*, *amh*, *foxl2*, *sox9*, *cyp19* and *ar* altered in brain tissue. Down-regulation of sex-related genes in gonad tissue. Female-skewed sex ratios and intersex characteristics (see Table 3).	Dietary exposure.	Fajkowska et al. (2021)
Nile Tilapia					
Nile Tilapia (larvae; weight = 6-8 mg)	Genistein	500 mg/kg	No differences in growth performance, survival, or sex ratios.	Dietary exposure.	Akinwande et al. (2011)
Nile Tilapia (mean weight = 0.01 g)	Genistein Daidzein	10, 20, 30 mg genistein/diet 10, 20, 30 mg daidzein/diet	(see Table 2-4)	Dietary exposure. Diet treatments also had 60 mg MT[j]/kg diet.	El-Sayed et al. (2012)
Nile Tilapia (juvenile; initial weight = 10.47 ± 1.24 g)	Genistein	0, 30, 300, and 3,000 µg/g	No differences in body composition, growth performance and survival. Lower digestive enzyme activities in all tissues except stomach in 3,000 µg/g diet. Lower stomach protease and intestine amylase activities in 300 µg/g diet.	Dietary exposure.	Chen et al. (2015)

Siamese Fighting Fish (Bettas)					
Male Bettas (adults)	Genistein Equol β-sitosterol	1, 10, 100, and 1,000 µg genistein/L 10, 100, and 1,000 µg equol/L 10, 100, and 1,000 µg β-sitosterol/L	Reduced activity and response latency time of 1,000 µg equol/L vs. control. Significant dose-dependent effects of genistein and equol on opercular display duration. No effect on survival or nest building.	Waterborne exposure. 17β-E2 treatments and negative (ethanol) control used.	Clotfelter & Rodriguez (2006)
Male Bettas (about 1 year old; 0.95-2.93 g; 32.82-43.70 mm)	Genistein β-sitosterol	1, 1,000 µg genistein/L, 1 µg β-sitosterol, 1 µg genistein/L + 1 µg β-sitosterol/L	No differences in circulating hormone steroids except lower 11-KT[k] in 1,000 µg genistein/L vs. 1 µg genistein/L. No differences in reproductive metrics.	Waterborne exposure. Positive (17β-E2) and negative (ethanol) controls used.	Stevenson et al. (2011)
Female Bettas (≥1 year old; 0.37-1.46 g; 24.33-38.85 mm)	Genistein β-sitosterol	1, 1,000 µg genistein/L, 1, 1,000 µg β-sitosterol, 1 µg genistein/L + 1 µg β-sitosterol/L	Reduced opercular display duration of 1,000 µg β-sitosterol vs. negative controls. No differences in any other metric.	Waterborne exposure. Positive (17β-E2) and negative (ethanol) controls used.	Brown et al. (2014)
Goldfish					
Goldfish (initial weight = 6.4-13.4 g)	Genistein Daidzein Coumestrol Equol	47,680 and 390,800 ng genistein/g diet, 41,120 and 416,800 ng daidzein/g diet, 226 and 1,325 ng coumestrol/g diet, 6.4 and 117 ng equol/g diet	No changes to growth or fish condition metrics. Higher VTG levels when feed ornamental carp feed.	Dietary exposure via ornamental carp and commercial trout feeds.	Ishibashi et al. (2002)
Goldfish (20 weeks old; mean weight = 2 g)	Genistein Daidzein	0:0, 24.263:21.702, 51.566:46.126, and 75.833:67.821 genistein:daidzein (µg/g)	Increased final body weights in highest diet for both sexes. Decreased GSI[l] values for both sexes at last two sample periods. Dose and time effects of E2 and T levels in both sexes.	Dietary exposure.	Bagheri et al. (2014)
Other species					

Yellow Perch (initial weight = 13-15.5 g)	Genistein	0.75 and 7.5 mg genistein/g diet	Reduced length growth of females fed 7.5 mg/g diet.	Dietary exposure.	Ko et al. (1999)
White bass (120 and 60-100 dph)	Genistein	0, 2, 4, and 8 mg genistein/g diet	No differences in growth or reproductive development factors. Higher VTG in 2 and 8 mg/g diets.	Dietary exposure. Two feeding trials performed.	Pollack et al. (2003)
Fathead Minnow (40-45 mm)	Genistein	0, and 10-1,280 µg genistein/L	No differences in survival, growth, or body symmetry metrics.	Waterborne exposure.	Ingham et al. (2004)
Walleye (mean weights; male = 1.31 kg, female = 3.14 kg)	Genistein	10^{-9} to 10^{-2} molar-based solutions	Reduced spermatozoa motility time, motility rank, and ATP content. Reduced fertilization rates.	Waterborne exposure.	Green & Kelly (2008)
Guppy (larvae)	Genistein	1 g genistein/kg diet	No difference in survival. Sex ratio-70.6% male, 24.8% female, 4.6% intersex individuals.	Dietary exposure.	Chakraborty et al. (2012)
Southern Flounder (84 dph; weight ≈ 0.3g)	Genistein	0, 100, and 1,000 g/kg diet	Increased female sex ratios in both genistein treatments. Lower survival rates in 1,000 mg/kg diet after grow-out period. Lower growth in 1,000 mg/kg diet after exposure period.	Dietary exposure. Exposure period of 69 days followed by 132 day grow-out period.	DiMaggio et al. (2016)

[a] X = times.
[b] VTG = vitellogenin.
[c] SPC = soy protein concentrate
[d] E2 = estradiol.
[e] DC_{50} = concentration of radio-inert competitor competing for 50% of bound [2,4,6,7-^{3}H]estradiol.
[f] TBARS = thiobarbituric acid reactive substance.
[g] [^{3}H]E2 = [2,4,6,7-^{3}H]estradiol.
[h] dph = days post hatch.
[i] ATP = adenosine triphosphate.
[j] MT = 17α-methyltestosterone.
[k] 11-KT = 11-ketotestosterone.
[l] GSI = gonadosomatic index.

Appendix C. Analytical information on the sample processing and analysis of biological samples collected as performed by the Unites States Geological Survey, Columbia Environmental Research Center (CERC) to determine genistein levels.

Analytical Method Information

Phytoestrogen Solid Method: Genistein Tissue Extraction

Prepared: 03/10/2022 by Kathy Echols of CERC

(1) Grind/Chop/cut up small fish with shears/knives onto rinsed aluminum foil. Transfer homogenized samples back into original sample vials/tubes. Rinse cutting and transfer instruments with DI water/MeOH and Acetone. Weigh out 100-500 mg of tissue into glass scintillation vials.

(2) Prepare for each set of samples – 1 matrix spike, 1 procedure spike, 1 matrix blank, 1 procedure blank, replicates (triplicate analysis of one sample per set); matrix suppression sample/spike. For the matrix suppression spike use one of the larger mass reference fish from the study and the minnow matrix tissue to extract and process with a set of samples. The suppression spike is not spiked initially but only before the LC/MS analysis to determine the matrix suppression effects in the LC/MS. A suppression correction factor was calculated from this and applied to the LC/MS data.

(3) To each scintillation vial with tissue add 1mL/100 mg of citrate buffer (25mmol/L pH5.0). For example, if a sample is 450 mg the volume of buffer added would be 4.5 mL.

(4) With the homogenizer – thoroughly homogenize the tissue and buffer mixture. Rinse the homogenizer between samples with DI water/MeOH.

(5) For each sample prepare a 15 mL centrifuge tube with 800 µL of MeOH. Write the ID/label directly onto the tube. Use the 2nd set of labels for the culture tubes in the SPE step below. Spike each with 50 ng of D4-genistein internal standard. For matrix spike/procedural spike QC samples, spike 100 ng (100 µL of 1 ng/µL standard) of native in addition to the internal standard.

(6) Re-homogenize or vortex the tissue/buffer mixture. Subsample the homogenized sample with 200 µL (20 mg equivalent) of the buffer/tissue and spike into the

centrifuge tube. Rinse the homogenizer with MeOH, DI water, acetone between samples.

(7) Sonicate these samples for 10 min at 25°C.

(8) Add 2 mL citrate buffer to each sample tube.

(9) Spike sample tubes with 100 µL of 1:50 dilution or 20 µL of the 1:10 dilution of *Helix pomatia* enzyme solution into each culture tube. Mix well.

(10) Incubate the samples at 37°C for 1 h.

(11) Centrifuge in Sorvall RC 3C Plus instrument for 20 min at 4500 RPM. Use the purple tube containers, balance the samples in the rotor before starting. Rotor is set to 20, temperature is 25°C, rpm is 4500, time is 20 min.

(12) Purify the genistein on SPE using 30 mg OASIS HLB cartridge. Activate cartridge sequentially by washing with 1 mL MeOH, 1 mL water (UP grade), 1 mL citrate buffer. Load the supernatants onto the cartridge. Wash with 1 mL water (UP grade). Elute the genistein with 2 mL of MeOH; collect in our standard culture tube.

(13) Reduce the methanolic extracts to ~100 µL with nitrogen blowdown/evaporator. Waterbath at 50°C. Transfer to conical HPLC vials with 3 x 50 µL rinses of MeOH. Evaporate to dryness in the vial and then add 12 µL of LCMS grade ACN and add 38 µL of LCMS grade water. Vortex well and move on to LC/MS.

Analytical Method Information

Method: UHPLC-MS/MS Parameters

Prepared: 03/10/2022 by Kathy Echols of CERC

A Waters Acquity-H UHPLC Class LC system controlled by MassLynx Version 4.2 Software (Waters Incorporated, Taunton, Massachusetts) coupled to a Waters Xevo TQ-XS with Stepwave Triple Quadrupole Mass Spectrometer were used for analysis. A Waters Cortecs® C18 (100 mm x 3.0 mm, 2.7 µm particles) column (Waters Incorporated, Taunton, Massachusetts) was used for chromatographic separation of target compounds at 30°C.

Mobile phase A consisted of LC/MS grade H_2O with 0.1% formic acid. Mobile phase B consisted of LC/MS grade acetonitrile with 0.1% formic acid. A gradient was used starting at 25% B, then increasing to 30% B, from 0 to 7 min, then increasing to 90% B from 7 to 9 min, holding at 90% B for 2 min, then decreasing back to 25% B for 2 min with a final hold at 25% B for 2 more min for a total run time of 15.0 min. A flow rate of 600 µL min^{-1} and a sample injection volume of 10 µL were employed. For the mass spectrometer conditions, a heated electrospray ionization source in positive mode was used to detect the targeted compound. The parameters for the mass spectrometer were set as follows: the capillary voltage was optimized at 4.00 kV; cone voltage was set at 20 V; desolvation temperature was set at 250°C; the source temperature was set at 150°C. All optimized collision energies, precursor ions and fragment ions shown below.

Parameters used for UHPLC-MS/MS analyses.

Compound	Chemical Formula	RT (min)	Precursor Ion	Collision Energy (eV)	Quantification Ion Transition (m/z)	Confirmation Ion Transition (m/z)
Genistein	$C_{15}H_{10}O_5$	4.48	271.112	26	153.022	215.075
D_4-Genistein [a]	$C_{15}D_4H_6O_5$	4.43	275.22	24	219.14	154.05

[a] Internal standard.

Quantification is by a 6-to-8-point calibration curve of genistein with d4 labelled internal standard at 50 ng. Genistein is determined by an internal standard method and are corrected for matrix suppression effects.

Appendix D. Analytical information on the sample processing and analysis of crude lipid levels in fish samples as performed by the Unites States Geological Survey, Columbia Environmental Research Center (CERC).

Analytical Method Information

Method: Crude Lipid Extraction and Analysis

Prepared: 03/10/2022 by Kathy Echols CERC

Total percent lipid determinations were done as follows. The samples were weighed, ground up, and dried by mixing with 4 times the mass of diatomaceous earth. Once mixed, the mixture was dried overnight. The sample was remixed and loaded into 22-mL stainless steel cells for the Dionex 200 Automated Solvent Extractor. QC included a procedure blank and a known fish matrix tissue. Samples were extracted with 95% methylene chloride and 5% acetone at a pressure of 1500 psi, 100°C for two 5-min extraction cycles. Pressurized extraction uses less solvent and takes less time. The solvent/lipid extract is collected in a 60-mL vial. The samples are brought up to a volume of 30 mL. The vial is then vortexed and 1.5 mL of this is transferred to a baked, pre-weighed shell vial. The solvent is baked off, the vial cooled and then reweighed. Percent lipid and mass of lipid are calculated from the initial sample mass, the percent sample used to determine the lipid, and the final lipid mass determined by subtracting the vial mass from the lipid plus vial mass.

Appendix E. Initial data needed to determine modelling parameters for both Level I and II fugacity models for Rainbow Trout fed a reference (Ref) diet or diets with genistein at 30.6 (NoGen), and 2,010 µg/g (HiGen) as treatments (Trt).

DOE	Microcosm Number	Trt	Total Fish Volume (mL)	Total Fish Weight (g)	Crude Lipid (%)	Microcosm Volume (L)	Mean Delivery (mL)
56	1	NoGen	120	119	6.00	45.0	1015
56	2	Ref	167	166	8.18	46.2	1007
56	4	HiGen	110	111	4.71	46.9	1017
56	5	HiGen	105	105	4.20	45.4	1005
56	7	Ref	161	162	6.80	45.8	1007
56	8	NoGen	110	112	4.95	45.8	1011
56	9	NoGen	115	117	6.47	47.7	1020
56	11	Ref	160	161	8.51	47.3	1017
56	12	HiGen	109	108	3.76	45.8	1014
56	13	Ref	135	133	7.52	46.6	1006
56	15	NoGen	125	126	5.74	47.3	1008
56	16	HiGen	119	120	6.21	45.4	1012
56	17	NoGen	125	124	6.14	46.9	1018
56	19	Ref	155	154	8.32	45.0	1002
56	20	Ref	165	166	9.61	45.8	1018
56	21	HiGen	112	111	5.40	45.4	1013
56	23	HiGen	110	110	6.14	43.9	1019
56	24	NoGen	115	113	5.94	43.9	1012
112	1	NoGen	245	256	9.13	45.0	1015
112	2	Ref	395	401	10.10	46.2	1007
112	4	HiGen	245	245	8.04	46.9	1017
112	5	HiGen	235	240	6.80	45.4	1005
112	7	Ref	375	390	9.22	45.8	1007
112	8	NoGen	243	238	8.19	45.8	1011
112	9	NoGen	255	263	9.71	47.7	1020
112	11	Ref	380	389	10.67	47.3	1017
112	12	HiGen	235	241	6.67	45.8	1014
112	13	Ref	330	333	13.53	46.6	1006
112	15	NoGen	280	284	11.60	47.3	1008
112	16	HiGen	255	258	10.80	45.4	1012
112	17	NoGen	260	268	9.52	46.9	1018
112	19	Ref	370	380	15.84	45.0	1002
112	20	Ref	375	385	14.31	45.8	1018
112	21	HiGen	245	250	18.61	45.4	1013
112	23	HiGen	230	235	10.44	43.9	1019
112	24	NoGen	250	256	10.75	43.9	1012

Appendix F. Analytical information on the sample processing and analysis of water samples as performed by the Unites States Geological Survey, Kansas Water Science Center, Organic Geochemistry Research Laboratory (ORGL) to determine phytoestrogen levels.

Analytical Method Information

Phytoestrogen Direct Inject Method: LCHM_DIM_PE

Prepared: 02/22/2021 by OGRL staff

The direct inject phytoestrogen ultra-performance liquid chromatography/tandem mass spectrometry (UPLC/MS/MS) method for the analysis of biochanin A, coumestrol, daidzein, equol, formononetin, and genistein in water was developed at the Kansas Water Science Center, Organic Geochemistry Research Laboratory (OGRL) and was adapted from a 37-compound solid-phase extraction UPLC/MS/MS method (Yost et al., 2014). Water samples were shipped overnight on ice to OGRL and stored at -20°C until extraction.

Water samples were analyzed for the 6 phytoestrogens by UPLC/MS/MS with electrospray ionization (ESI) and multiple reaction monitoring (MRM) with Daidzein-D_3 and Genistein-D_4 as surrogate internal standards. Standard curve, check standards, and laboratory blank samples were prepared in Type I water. A duplicate sample, laboratory blank, and check standards were analyzed after every tenth sample. All standards, laboratory blanks, and check standards were treated the same as the environmental water samples. Standard curve solutions were prepared at concentrations of 5, 10, 20, 50, 100, 200, 500, 1,000, 5,000, and 10,000 ng/L, check standards were prepared at 50 and 1,000 ng/L, the standard addition samples were spiked at 1,000 ng/L, and the surrogate internal standards were spiked at 1,000 ng/L for Daidzein-D_3 and 6,600 ng/L for Genistein-D_4.

Samples, standards, and laboratory blanks were prepared for analysis by taking 4 mL aliquots of water or sample in 4-mL glass vials, spiking with the surrogate internal standards, and adding appropriate amounts of the standard mix to standards and standard addition samples. The prepared samples were filtered with a 0.2-micron GHP 13mm syringe filter (Waters Corp., Milford, Massachusetts) with 1.5 mL used to condition the syringe filter and 1.5 mL collected for analysis in 2-mL Agilent silanized screw top vials (Agilent, Santa Clara, California). Samples were stored at -20°C until analysis.

The samples were analyzed using a Waters Acquity H-class Bio UPLC with a Sciex (Framingham, Massachusetts) API 5500 Triple Quadrupole Mass Spectrometer. For separation a vanguard pre-columns and Waters Acquity BEH 1.7 µm, 50x2.1 mm UPLC analytical column were used with a (A) water, (B) methanol, and a post-column infusion of 10 mM ammonium hydroxide mobile phase. MS positive-ion mode was used with electrospray ionization and two multiple reaction monitoring (MRM) transitions were selected for each analyte and identification was based on the retention time and the ratio of the two MRM transitions being within +/-25%. A linear 1/x weighting was applied to the calibration curve and detected compounds were quantitated using standard addition calculations. To meet quality control specifications environmental samples and duplicates did not exceed a 20% difference, the target matrix spike recovery range was within 80-120% for analytes with matching surrogate internal standards, and the check standard accuracy was within 80-120%. The reporting limit was 10 ng/L for biochanin A, 20 ng/L for coumestrol, equol, formononetin, genistein and 50 ng/L for daidzein.

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