

The Effects of the Soy Isoflavone Genistein on the Reproductive Development of Striped Bass

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Abstract.—The aquaculture industry has made great strides toward developing diets for finfish that provide adequate nutrition for the animals being maintained while minimizing the cost to the farmer. Alternative, plant-derived protein sources are used to minimize cost. Soybean meal is the plant protein most commonly used because it maintains adequate growth. However, soy is rich in phytoestrogens, the most abundant of which is genistein. Studies have shown variable physiological effects associated with exposure to genistein. The objective of this research was to investigate the effects of feeding genistein to juvenile striped bass *Morone saxatilis* at varying concentrations in the diet. The first experiment was designed to determine whether juvenile striped bass are capable of responding to estrogen exposure. Juvenile striped bass were given weekly injections of estradiol benzoate for 3 weeks and showed a significant response to the estrogen via expression of vitellogenin. In two additional experiments, genistein was fed to striped bass fingerlings (approximately 120 d old and 60–100 d old) in varying concentrations (0, 2, 4, and 8 mg/g of diet) to determine whether a similar response would be elicited. No significant differences were found among the growth variables and histological endpoints, although a significant vitellogenin response was observed at the 2-mg/g and 8-mg/g doses. These results show that the juvenile striped bass did respond to the estrogen-like function of genistein and that the genistein induced a U-shaped response curve that is characteristic of the low-dose effects of some endocrine-disrupting chemicals.

One of the main goals of the aquaculture industry is to provide the best quality feed ingredients to optimize the health, growth, and reproductive performance of fish while reducing the cost of production. One option for achieving this goal is to use plant proteins as a substitute for more costly animal proteins. As such, soybean meal has received considerable attention. At times, soy protein may constitute 40% of the diet, and while soybeans *Glycine max* are an excellent source of nutrition (Gallagher 1994) they contain natural phytoestrogens known as isoflavones. Soy isoflavones have varying potencies in comparison with estrogen. Genistein is the most potent, at 1,000 times less activity than estradiol (Knight and Eden 1996). Genistein is also the most abundant and most biologically active of the soy isoflavones and has been shown to exert a weak estrogenic effect

on the behavior of rats if exposure occurs during sexual differentiation (Flynn et al. 2000). Estradiol is critical for sexual differentiation and reproductive function; therefore, genistein may produce more subtle effects than estradiol. Possible effects are competition with endogenous hormones and the stimulation of a response at an inappropriate developmental stage (Knight and Eden 1995). Sexual differentiation or organization of the reproductive axis in vertebrates relies on many endogenous factors, including steroid hormones. In addition, exogenous environmental cues and stimuli may affect this process. As the juvenile fish matures, steroids exert predominantly stimulatory effects on the synthesis of the gonadotropic hormone GtH II, which in turn begins the activation of the reproductive system or sexual maturation (Holland 1999). In this regard, exposure to genistein in diets containing soy protein may exert subtle effects that are not detectable in adult organisms. However, due to the action of the isoflavone and the plasticity of the system during the organization of the re-

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Received August 28, 2002; accepted March 12, 2003

productive axis, younger organisms may be much more susceptible.

Vitellogenin is an estrogen-inducible precursor to yolk protein that is produced in the liver (Mommsen and Walsh 1988) and an excellent biomarker for exposure to estrogenic substances (Kime et al. 1999). Vitellogenin has been purified and characterized both biochemically and immunologically in mature striped bass *Morone saxatilis* (Sullivan et al. 1991; Tao et al. 1993). Detectable levels of vitellogenin in the blood plasma of males, juveniles, and nonvitellogenic females are indicative of exogenous exposure to estrogen.

The results from previous studies involving genistein and other phytoestrogens in the diet vary among different species of teleost fish. Dietary soybean and purified phytoestrogens produced large changes in the plasma vitellogenin levels of Siberian sturgeon *Acipenser baeri* (Pelissero et al. 1991a, 1991b), and feeding genistein to yellow perch *Perca flavescens* compromised growth in females (Ko et al. 1999).

If dietary genistein affects growth or reproductive development in young striped bass, these effects may be detected during sexual differentiation, when exogenous estrogen might also induce an effect on normal developmental processes. The purpose of this study was to determine the effects of genistein as a exogenous estrogen mimic that is found naturally in the diets of striped bass in aquaculture. Fingerling striped bass were challenged with estradiol benzoate (EB) to determine whether a vitellogenin response could be induced by a known estrogen during this young, relatively labile stage of development. The results would then indicate whether animals of this age are susceptible to exogenous substances that affect normal hormonal processes. Dietary genistein was then fed to fingerling striped bass to determine whether a vitellogenin response can be induced by this weaker substance as well.

Methods

Estradiol Challenge

A total of six striped bass fingerlings (approximately 120 d old) from the Crane Aquaculture Facility (CAF) in Baltimore, Maryland, were used in this study. According to methods described by Woods et al. (1995), fish were anesthetized in a 70-mg/L concentration of quinaldine and injected intraperitoneally with a 5-mg/kg dose of EB stock solution. The EB injections were repeated at weekly intervals for three subsequent weeks. Blood was

collected in the fourth week, following the final estradiol injection. Blood was taken using heparinized needles inserted into the caudal vasculature while fish were anesthetized. Aprotinin (a protease inhibitor) at a concentration of 1 total international unit/mL or 80 μ L was added to the microcentrifuge tube per milliliter of blood collected and then centrifuged for 5 min at 10,000 revolutions per minute at 4°C. Plasma was then transferred to microcentrifuge tubes and stored at -180°C until further analysis. The induction of vitellogenin by EB was verified with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis (Tao et al. 1993).

Dietary Genistein Experiment I

Striped bass fingerlings (approximately 120 d old) were randomly placed into 12 tanks (200-L capacity) in a flow-through system at the CAF. Fish (6/tank) were weighed and measured at the beginning of the study before being fed the experimental feed. Fish in each of the 12 tanks were randomly assigned one of four experimental diets in triplicate and fed 1% of their body weight per day (BWD) for 6 weeks. Fish were kept on a 24-h light cycle to maximize consumption of the diet. At the end of the study period, all fish were weighed and their total length measurements recorded. Fish that showed a significant increase in body weight were euthanized in buffered MS-222 (tricaine methanesulfonate; 200 mg/L). This increase would indicate that the fish were consuming the diet at the 1% BWD rate at which they were fed and therefore gained weight. The effects of sexually dimorphic growth would not be a factor until the third or fourth year of life (Woods et al. 1999). Blood samples were collected from 24 randomly selected fish ($n = 6/\text{treatment}$) and processed as described previously. Tissue samples (gonad and liver) were weighed to obtain gonadosomatic index (GSI) and hepatosomatic index (HSI) values (organ weight/body weight $\times 100$). Gonadal tissue samples were then placed in Bouin's tissue fixative for histological analysis, and liver samples were stored at -80°C.

Diet preparation.—A commercial striped bass diet, Bass Brood (Ziegler Brothers, Inc.), was prepared with varying levels of purified genistein (Toronto Research Chemicals, Inc., North York, Ontario). Genistein dissolved in 75 mL ethanol was added to the diet at concentrations of 0, 2, 4, and 8 mg/g. The feed was mixed in a Hobart mixer in 2,000-g portions for 7–10 min with 15% water added. The experimental diets were prepared with

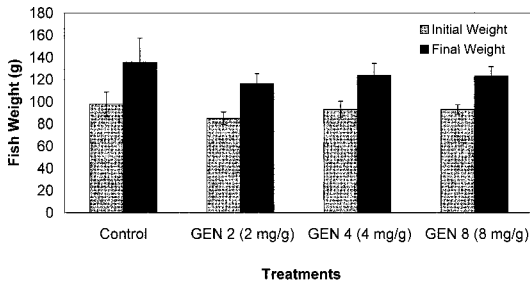


FIGURE 1.—Initial and final body weights (mean \pm SE) in dietary genistein experiment I. Numerical values expressed as milligrams per gram refer to the concentrations of genistein (GEN) in fish diets; $n = 6$ fish/treatment.

a standard pelleter. Before preparing each diet, 0.5 kg was run through the pelleter and discarded to remove any residue from the previous diet pellet. The diets were mixed from low to high levels of genistein to minimize the overlap in genistein concentration. They were air-dried overnight and then stored in a freezer.

Histological preparation.—Tissue was dehydrated in increasing concentrations of ethanol (70% to 100%) for various time increments. It was then transferred to four different solutions with varying concentrations of chloroform. Melted Paraplast was used to embed the tissue, and the blocks containing it were allowed to cool overnight to harden. The blocks were then cut into sections 5 μ m thick, mounted, and dried. The tissue was stained with a combination hematoxylin and eosin stain according to the methods of Hinton (1990) and W. Kuenzel (Department of Animal and Avian Sciences, University of Maryland, personal communication).

Vitellogenin assay.—Plasma samples were as-

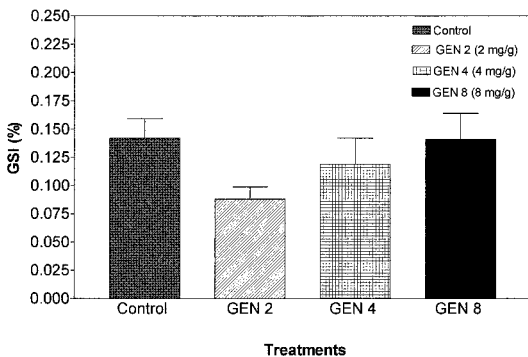


FIGURE 2.—Gonadosomatic index values (GSI = $100 \times$ weight of gonads/body weight; +SE) for fish in the different genistein treatments; $n = 24$ fish.

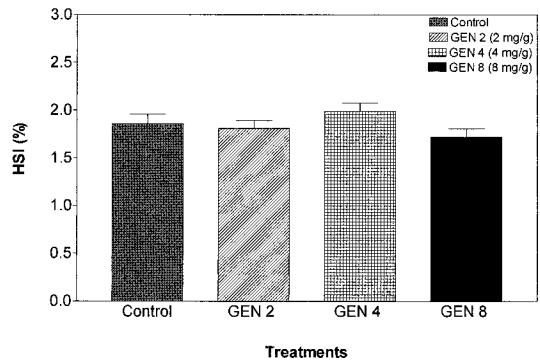


FIGURE 3.—Hepatosomatic index values (HSI = $100 \times$ liver weight/body weight; +SE) for fish in the different genistein treatments; $n = 6$ fish/treatment. A normal pattern of liver development is indicated.

sayed using a specific enzyme-linked immunosorbent assay to detect striped bass vitellogenin (Heppell et al. 1999). Triplicate samples were diluted 1:1,000 with primary antibody solution on 96-well plates. Wells were coated with buffered bovine serum albumin (used to measure nonspecific binding) and then blocked using buffered normal goat serum. An interassay pool of plasma was also included in the wells as a control. Standards were made by serial dilution of a stock solution of purified striped bass vitellogenin in phosphate-buffered saline (PBS). Plasma samples were incubated and washed several times with PBS, and a secondary antibody (goat antirabbit serum diluted in PBS) was added. Enzyme substrate (peroxide and peroxidase [enzyme number 1.11.1.7; IUBMB 1992]) was added to each well and incubated for 5 min, then the reaction was terminated with the addition of H_3PO_4 . Absorbance was read at 450 nm on a microplate reader. The upper and lower limits for sensitivity of the assay are 1,188 and 33 ng/mL, respectively (Heppell et al. 1999).

Statistical analyses.—Growth data (GSI, HSI, and body weight measurements) were analyzed by means of the general linear model procedure followed by the Student–Newman–Keul test for mean separation. Vitellogenin was analyzed by means of mixed analysis of variance (ANOVA) and Tukey's least-squares means test ($P \leq 0.05$ determined significance). Chi-square analysis was used to verify the sex ratios for the treatment groups after verification of the sex of individual fish via histological analysis. All analyses were performed using SAS for Windows (SAS Institute 1999).

Dietary Genistein Experiment II

Experimental animals.—All fish were full siblings and were fed a basal diet for 1 week before

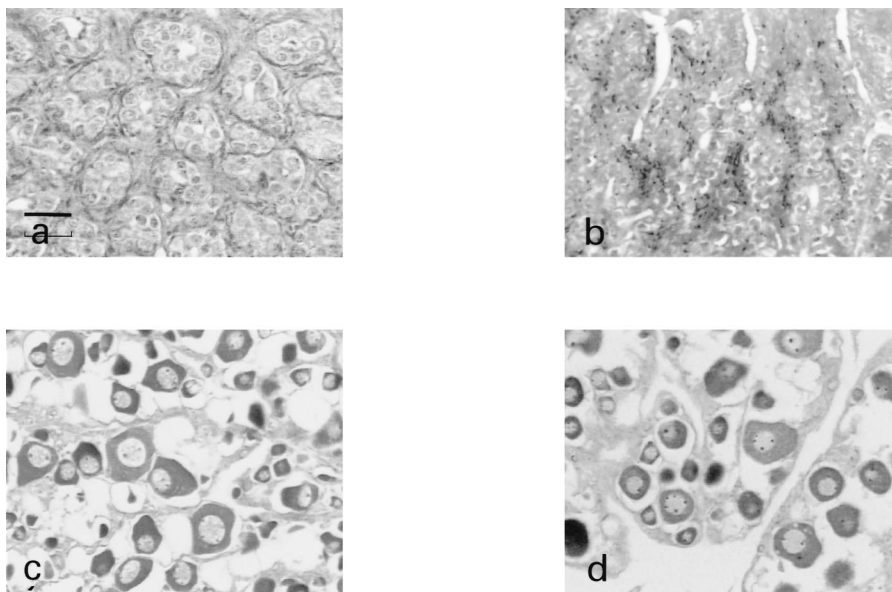


FIGURE 4.—Cross sections of gonads from (a) an immature male striped bass from the control group (bar = 24 μm), (b) an immature male from the 2-mg/g treatment group, (c) an immature female from the 4-mg/g treatment group, and (d) an immature female from the 8-mg/g treatment group. All fish were 120 d old; $n = 6$ fish/treatment.

the beginning of the study so that they would acclimate to the diet and the environment. The study began 60 d posthatch (dph).

Striped bass fingerlings ($N = 360$) from the population at the CAF were used in this study. Fish were weighed in groups of 30 to obtain a mean weight per fish, and then each group was randomly placed into 1 of 12 tanks (200-L capacity) in a flow-through system. Each tank contained 30 fish and was randomly assigned to a dietary treatment. The experimental design was as previously described. One midstudy sampling was done at 3 weeks to weigh and measure fish to obtain body measurements and adjust the percent BWD calculations. At the end of the 6-week period, all of the fish were weighed, measured, and fin-clipped. Fin clips were assigned to treatment groups and to replicates within a treatment for identification purposes as fish were moved into a communal, 2-m-diameter tank on a recirculating system. The fish were maintained on a basal diet in the communal tank for an additional 6 months until they reached approximately 100 g (at 9 months of age). During this time, body weight measurements and total lengths were recorded at 6-week intervals to obtain growth data and determine when fish were large enough to sample. Fish were moved to this communal tank to standardize water quality and light and feed conditions. A total of 24 fish ($n =$

6/treatment) were then randomly chosen and euthanized with an overdose of buffered MS-222 (200 mg/L) to obtain tissue and blood samples as previously described. Final length and weight measurements were taken for all fish. The diet preparation, sample processing and analyses, and statistical procedures were all performed as previously described. Pairwise mean comparisons were performed to compare the growth data means by date for the intervals from the beginning of the study until sacrifice.

Results

Estradiol Challenge

The production of vitellogenin was observed in five of the six fish experimentally challenged with EB (samples 1, 2, 4, 5, and 6; results not shown). In addition to the experimental samples, samples run on the gel from an estrogenized female and a male striped bass were used as controls. The bands for vitellogenin were not found in sample 3 or the estrogenized male. A Western blot was done to validate the SDS-PAGE analysis as a positive control; it showed results similar to those of the SDS-PAGE.

Dietary Genistein Experiment I

No significant differences in body weight gain were found between the controls and any of the

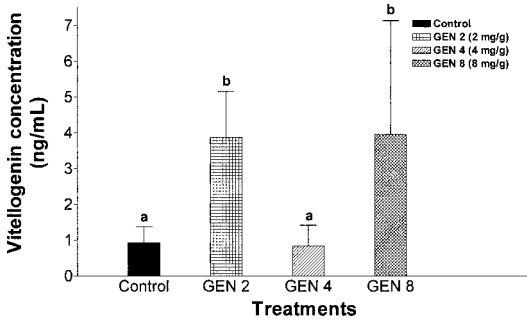


FIGURE 5.—Vitellogenin concentrations (mean + SE) in the different genistein treatment groups ($n = 6$ fish/treatment) as determined by enzyme-linked immunosorbent assay. Means with different lowercase letters are significantly different ($P \leq 0.05$).

genistein treatments (Figure 1). Fish treated with genistein had GSI and HSI values similar to those of the controls, so there were no significant differences with respect to the gonadosomatic and hepatosomatic index values (Figures 2, 3). Figure 4 shows representative histological sections of tissue from each treatment group. A chi-square analysis of histological samples verified an average 1:1 sex ratio in all treatment groups. Primary spermatocytes were observed in the testes of immature striped bass (Figure 4a, b). Immature oocytes can be seen in the ovary of the immature female (Figure 4c, d). Significantly higher levels of vitellogenin were found in both the 2-mg/g and 8-mg/g treatment groups compared with the control group ($P \leq 0.05$). The 4-mg treatment group did not differ significantly from the control. The plasma vitellogenin of the 8-mg and 2-mg groups was significantly greater than that of the 4-mg group (Figure 5).

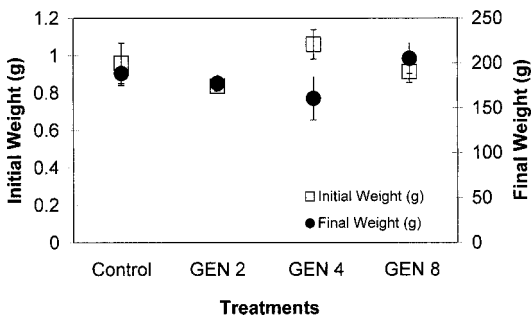


FIGURE 6.—Initial (60-d-posthatch) and final (270-d-posthatch) body weights (mean \pm SE) of fish exposed to dietary genistein from 60 to 100 d posthatch; $n = 90$ fish/treatment.

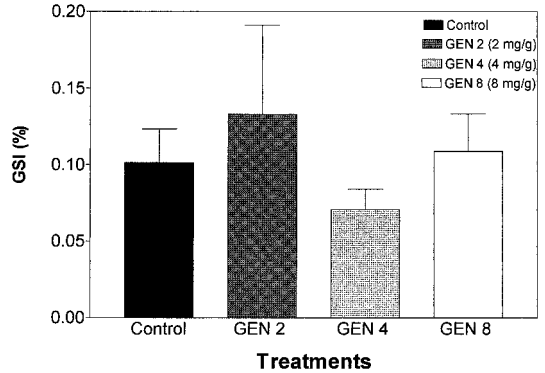


FIGURE 7.—Gonadosomatic index values (mean + SE) for fish fed different concentrations of genistein from 60 to 100 d posthatch; $n = 6$ fish/treatment. No significant differences were observed among means.

Dietary Genistein Experiment II

The mean body weight of the fish in this experiment increased by a factor of more than 1,000, which is the expected growth pattern for juvenile striped bass (Figure 6). No significant differences were observed in gonad or liver tissue growth (Figures 7, 8). Figure 9 shows representative sections of gonadal tissue from each treatment group. Distinct primary oocytes were observed in the histological sections shown in Figure 9a and 9b; these oocytes lack the lipid globules characteristic of mature oocytes. Primary spermatocytes are visible in Figure 9c and 9d, but no mature spermatids were seen in these sections, indicating that these animals were reproductively immature (yet developing normally). The sex ratio observed in these fish was 1:1 ($n = 24$). A significantly lower percentage body weight gain (data not shown) was observed in the 4-mg/g treatment group. Pairwise mean comparisons of the growth data obtained from the

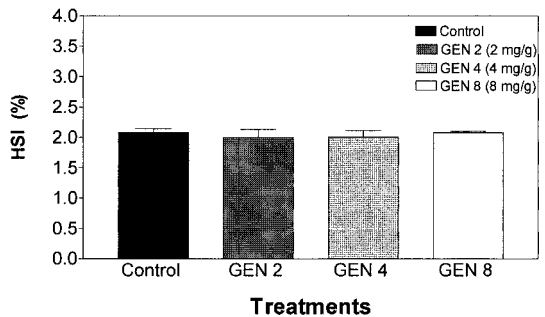


FIGURE 8.—Hepatosomatic index values (mean + SE) for fish fed different concentrations of genistein from 60 to 100 d posthatch; $n = 6$ fish/treatment. No significant differences were observed among means.

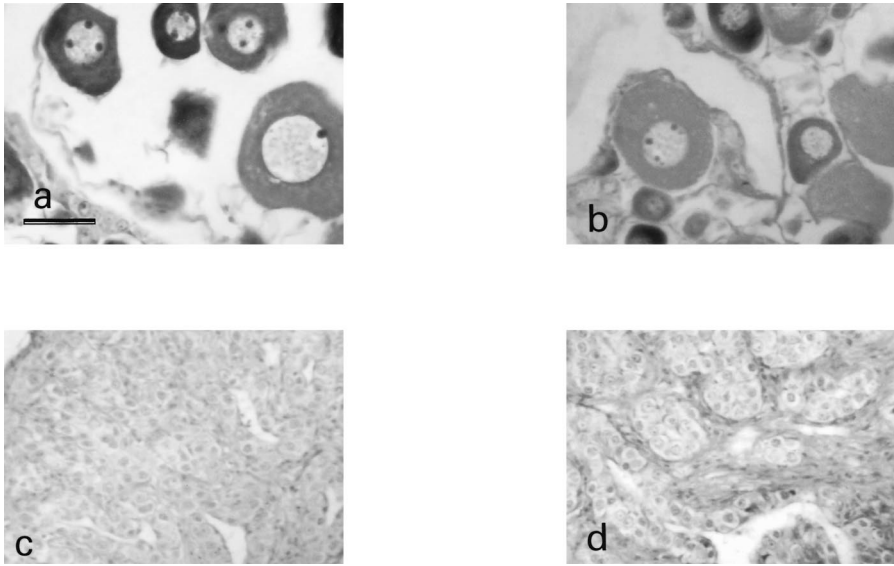


FIGURE 9.—Cross sections of gonads from (a) an immature female striped bass from the control group (bar = 40 μ m), (b) an immature female from the 2-mg/g treatment group, (c) an immature male from the 4-mg/g treatment group, and (d) an immature male from the 8-mg/g treatment group. All fish were 270 d old; $n = 6$ fish/treatment.

6-week interval measurements showed that this group differed significantly from the control group on the 211th and the 253rd days after the end of the study but prior to sacrifice. The 4-mg treatment group also differed significantly from the 8-mg group on day 253. Vitellogenin analysis produced a U-shaped plot (Figure 10). Fish in both the 2-mg and 8-mg groups had significantly higher vitellogenin responses than those in the control and the 4-mg groups but were not significantly different from one another.

Discussion

Vitellogenin is a valuable biomarker for assessing exposure to estrogenic substances in the environment. It may also be used to assess normal sexual maturation in female oviparous vertebrates because it is estrogen dependent (Brooks et al. 1997; Sullivan et al. 1997; Tyler et al. 1999). In addition, vitellogenin production is indicative of estrogen exposure in immature females as well as males, since only mature females normally produce this protein (during oocyte development). The results from the estradiol challenge indicate that striped bass as young as 4 months of age are capable of producing vitellogenin. These data provide evidence that the physiological system of a striped bass of this age is capable of recognizing an exogenous estrogenic substance and of reacting by producing vitellogenin.

Vitellogenin production occurred with exposure to genistein, which is of interest because genistein is several orders of magnitude less potent than endogenous estradiol (Knight and Eden 1996) and yet was sufficient to induce a response. The results from the histology and chi-square analysis did not show alterations in sex ratios or gonadal development, as normal development patterns were observed for both primary spermatocytes and primary oocytes. Although a vitellogenin response can be sex related (e.g., a lower response in one treatment may indicate more male fish in that treatment), the histological observations and chi-

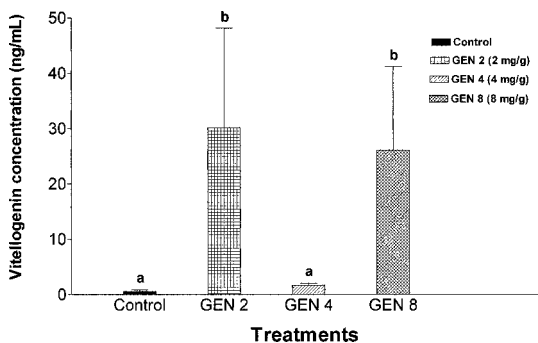


FIGURE 10.—Plasma vitellogenin concentration (mean + SE) in fish exposed to genistein from 60 to 100 d posthatch; $n = 6$ fish/treatment. Means with different lowercase letters are significantly difference ($P \leq 0.05$).

square analysis revealed a 1:1 sex ratio for all treatment groups. The levels of genistein utilized in this study did not affect the hepatic, gonadal, or normal somatic growth of these fish. The lack of discernable effects on gonadal differentiation and tissue growth provides evidence that the levels of genistein were not potent enough to have long-term effects on gonadal morphology. However, the vitellogenin response suggests that the timing and dose were sufficient to exert transient effects and that the physiological system of the fish recognized genistein as estrogenic.

The dose-related effects for vitellogenin concentrations are also of interest because of the U-shaped response curve. This type of curve indicates that genistein is acting on the endocrine system of juvenile striped bass in both a stimulatory and an inhibitory manner, depending on the level of the substance recognized by the system. Our results also suggest that the genistein levels in this study were too low to exert a classic dose-response effect because genistein is a weak estrogen mimic. The fact that the 2-mg/g treatment group showed increased vitellogenin production suggests that there was some stimulation through an unknown mechanism, which was followed by an inhibitory mechanism in the 4-mg/g group and a direct stimulatory effect in the 8-mg/g group (which suggests that the system was then being up-regulated from this level of genistein). This hypothesis is supported by the controversial function of genistein, which has been regarded as both an estrogen agonist and an estrogen antagonist (Holt 1998). A similar indirect treatment response curve has been discussed relative to the low-dose effects of endocrine-disrupting chemicals (Nagel et al. 1997; vom Saal et al. 1997). In these cases, in which exposure to diethylstilbestrol showed adverse reproductive effects in rat pups, an inverted U-shaped treatment effect was seen in low doses. In these studies, low levels of diethylstilbestrol stimulated prostate growth whereas higher doses were toxic to the organism and effectively diminished prostate size.

The specific timing of exposure in the life cycle of the organism may be just as crucial to the effects of a given substance as the dose administered. The 60–100-d age window was based on findings by Schutz and Harrell (1999) in which exposure to a potent steroid hormone resulted in the sex reversal of the population. Schutz and Harrell (1999) and Gomelsky et al. (1999) suggest that circulating steroid levels have the most influence on sexual differentiation in striped bass and its hybrids from

30 to 60 dph and 63–93 dph, respectively. It is interesting to note that similar vitellogenin results were obtained from both genistein studies discussed here, given that fish of different ages (120 dph for the initial experiment and 60 dph for the second) were exposed to the same genistein levels in the diet.

The use of soy products in fish diets introduces the issue of how much soy protein can be substituted for animal protein without causing a significant decline in growth and development. Although known levels of genistein were tested for significant changes in the growth and development of juvenile striped bass, these levels may not be biologically relevant to those found in diets containing soy protein. The genistein in these diets is in the form of glycoside, and the pure genistein used in this study may not reflect exactly what occurs once a diet is processed (Mahungu et al. 1999). Similarly, the levels utilized in this study were based on those in the studies done by Ko et al. (1999), which were similar to the isoflavone levels found in some finfish diets. Isoflavone levels in some soy protein concentrates approach 6,000 mg/kg (Mambriani et al. 1999). Our highest level of genistein (8 mg/g or 8,000 mg/kg) was used to provide a broad spectrum of levels. Our results showed that genistein levels of 0, 2, 4, and 8 mg/g had no adverse effects on body weight gain and liver and gonad growth when fed during a potentially vulnerable stage. The observed significant difference in percent body weight gain with respect to the intermediate level of genistein (4 mg/g) may reflect a delayed response in this treatment group. Significant differences were found with respect to body weight gain in this group by the 211th day after these animals were removed from the experimental diet and placed in a communal tank, which was 6 weeks prior to sacrifice. Thus, our results may not be comparable to those of Ko et al. (1999), who determined that growth was compromised in female yellow perch by exposure to 7.5 mg/g genistein (without a delayed effect). However, due to the lack of data on growth after day 253, it is not clear to exactly what extent genistein affected growth in this species at these levels.

Differences in the growth response to the genistein may be due to differences in fish species' ability to metabolize or utilize a particular substance to which it has been exposed in the diet. This would indicate that the effects of genistein on certain endpoints vary by species. Yet, the vitellogenin response has proved to be a valuable,

universal endpoint, as previously mentioned. Both male and female yellow perch showed a significant increase in plasma alkali-labile phosphoprotein phosphorus, which is indicative of vitellogenin induction, and Siberian sturgeon exposed to various isoflavones (including genistein) also showed a significant increase in plasma vitellogenin levels (Pelissero et al. 1991b).

Furthermore, in this study histological analysis determined that there were no adverse effects on gonadal cell differentiation. This could indicate that the levels used in this study were too low to induce overt effects on large-scale physiological and morphological mechanisms such as sex cell differentiation and therefore that the action of genistein as a weak estrogen mimic is another key to the observed effects on more subtle factors such as vitellogenin production. Overall, the following observations were made from the results of this study: (1) Genistein exposure in striped bass during the early fingerling stage did not affect the growth or development of the reproductive factors examined at the levels tested; and (2) a vitellogenin response was observed for two doses (2 and 8 mg/g) but there did not seem to be any effect on gonadal growth or cell differentiation.

Despite our failure to observe overt changes in the physiological response to the introduction of an exogenous estrogen during a potentially labile, juvenile stage of development, it may be that significant effects on sexual differentiation or development will not manifest themselves until the animal is sexually mature. Therefore, studies currently in progress have been undertaken to determine whether there are delayed effects on the reproductive development of striped bass. Of particular interest are the possible effects of genistein on aromatase activity, as genistein has been found to slightly inhibit this process in rainbow trout *Oncorhynchus mykiss* (Pelissero et al. 1996) and this may ultimately affect the interactions between the brain and gonadal function when the animal reaches sexual maturity.

Conclusions

Juvenile striped bass approximately 4 months of age were capable of producing vitellogenin as a result of EB injection, which we believe is the youngest age for this response ever reported in this species. However, feeding striped bass as much as 8 mg of genistein per gram of diet during periods of rapid growth did not have adverse effects on body weight, GSI, or HSI. While capable of in-

ducing a vitellogenin response, these levels of genistein had no effect on gonadal cell differentiation.

Acknowledgments

This project could not have been done without the advice and guidance of the following people: Mahmoud Abdelnabi, Tony Pait, and Nichola Thompson. Special thanks go to Julie Hazelton, Shuyang He, Karen Jenkins-Keeran, Michael Quinn, Jr., Dan Thiesen, and Julie Wu for overall help with this project. We also thank Greg Weber for vitellogenin assay materials and technical assistance. Funding for this research was provided by Maryland Agricultural Experiment Station competitive grant AASC-00-34. All experiments were conducted under approved Institutional Animal Care and Use Committee protocols.

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