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Environmental Contamination and Toxicology

Effects of Genistein on Growth and Development of Aquatic Vertebrates

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Phytoestrogens are a class of compounds found in a variety of plants. They are structurally and functionally similar to the animal steroid hormone estrogen (Holmes and Phillips 1999) and, consequently, they disrupt endocrine function in animals by binding to hormone receptors in exposed organisms (Keeler and Panter 1989). Genistein, one of the most thoroughly studied phytoestrogens, is also known to interfere with cell proliferation by inhibiting tyrosine kinase activity (Akiyama et al. 1987). Genistein has been found to protect against some forms of cancer (Adlercreutz 1995); thus, most studies have been conducted to test for human health effects of prophylactic uses of phytoestrogens (e.g. soy-based foods, diet supplements).

Genistein and other phytoestrogens are also potential environmental contaminants, but their effects on wildlife populations are poorly understood. Genistein is released from bleached wood pulp into waters downstream from pulp and paper mills. The chemical has been shown to alter sex steroid levels and decrease reproductive capabilities of fish located near these mills (Kiparissis et al. 2001). Exposure to bleached pulp mill effluent is also known to reduce growth and affect stress responses in fish (Borton et al. 1996; Lappivaara et al. 2002), but little is known about whether these effects are due specifically to phytoestrogens.

The aim of our present study is to determine the effects of genistein on whole-organism characteristics of aquatic vertebrates. Specifically, we tested the hypothesis that acute exposure to genistein would decrease survival and impair growth, embryonic development, and developmental symmetry of two test organisms, the fathead minnow (*Pimephales promelas*) and the South African clawed frog (*Xenopus laevis laevis*).

MATERIALS AND METHODS

We obtained 40 – 45 mm juvenile minnows from a commercial supplier and allowed them to acclimate in 38-L glass aquaria filled with aged tap water for several days before the experiment began. Genistein (mg; Sigma-Aldrich Co.) was dissolved in ethanol (mL) at a 5:1 ratio (concentration of ethanol far below that at which deleterious effects are observed; L Crawshaw, *personal*

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communication). We mixed the genistein/ethanol solution into tanks to create nominal concentrations of 10 – 1280 μg/L (see below). Additional ethanol was added to each tank until all exposure tanks, including the control tank, had similar levels of ethanol. Minnows remained in these exposure tanks for 96 hr, with a 50% water change (and subsequent re-dosing) every 24 hr. Twenty minnows were in the control treatment and 12 minnows were in each genistein treatment.

Our initial exposure concentration was 10 μg/L, based on levels reported by Kiparissis et al. (2001), and increased by a factor of two. We observed no deleterious effect on survival at these concentrations (Figure 1a); therefore, we increased exposure concentrations to 80 – 1280 μg/L.

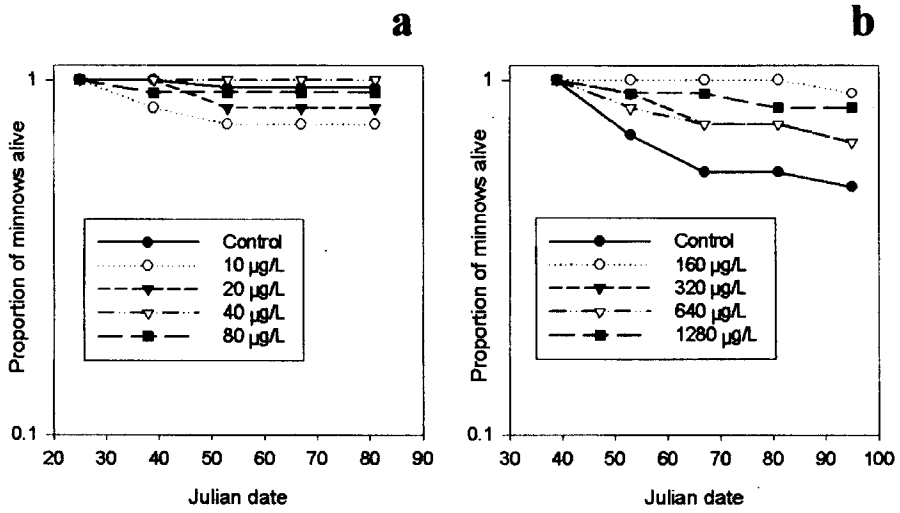
Following the exposure period, we separated minnows in 9-L tanks (6 fish per tank) and monitored growth and development for eight weeks. During the growth period, minnows were fed a daily ration (50% dried brine shrimp, 50% commercial fish flakes) equivalent to 4% of their body mass. Tanks were maintained at 22 °C and equipped with air diffusers. We weighed minnows (±0.01 g) every two weeks and adjusted food rations (for each 9-L tank) accordingly. After eight weeks, minnows were anesthetized with methane sulfonate and sacrificed. We used digital calipers to measure three independent, bilateral traits (±0.01 mm): post-orbital distance (eye to edge of gill cover) and pectoral and pelvic fin lengths. We made three replicate measurements on the right and left sides of each minnow and recorded the average of these measurements.

For the *Xenopus* exposure experiment, we obtained fertilized eggs from hCG-induced female frogs and standard *in vitro* fertilization techniques (Sive et al. 2000). Embryos were incubated in 0.1X MBS at 22 °C. Embryos were then assigned randomly to one of six treatments: control without ethanol, control with ethanol, and 80, 160, 320, and 1280 μg/L genistein. Genistein was dissolved in ethanol as described above. Sample sizes were 80, 80, 80, 80, 50, and 50 embryos, respectively. Embryos within a treatment were divided among three 50 mL Petri dishes. We monitored embryos for 48 hr (tail-bud stage), at which time developmental status was determined using the dorso-anterior index (DAI) of Kao and Elinson (1988).

Statistical analyses for the minnow experiment were conducted using SPSS (v. 11.0). We used logistic regression to determine the effect of genistein concentration on survival. Analysis of covariance (ANCOVA), with Julian date (0 – 365) as a covariate, was used to analyze minnow growth and trait symmetry following genistein treatment, with body length (mm) and mass (g) as covariates in the latter analyses. Differences were considered significant at $P < 0.05$. Bars on figures are means ± SE.

RESULTS AND DISCUSSION

Genistein is released in pulp and paper mill effluent, even after the bleaching



Figures 1a, b. Survival of juvenile fathead minnows exposed to genistein concentrations of (a) 10 – 80 µg/L and (b) 160 – 1280 µg/L for 96 hr. Survival was monitored for eight weeks following exposure.

and wastewater treatment processes, at concentrations high enough to cause reproductive impairment in fish (Kiparissis et al. 2001). The population implications of genistein exposure, however, are largely unknown. We exposed fathead minnows to genistein levels far exceeding those reported by Kiparissis et al. (2001) and, despite slightly lower survival of control fish, found no effect of exposure concentration on minnow survival after eight weeks post-exposure (logistic regression, $\beta = -0.001$, $P = 0.29$; Figures 1a, b).

Growth rate and developmental symmetry are important indicators of environmental contamination because of their impacts on fitness (Thornhill and Møller 1998). Many contaminants are known to interfere with the thyroid gland, and thus have the potential to affect growth rate in fish and other wildlife (Donaldson et al. 1979). Disruption of growth and development by anthropogenic chemicals is well documented (Colborn et al. 1993), but little is known about the effects of phytoestrogens. Our results show only marginal evidence for impaired growth in fathead minnows exposed to genistein. There was no significant dose-dependent effect of genistein on minnow body mass (ANCOVA; date: $F_{1,260} = 114.9$, $P < 0.001$, treatment: $F_{4,260} = 0.99$, $P = 0.41$), but minnows at the highest concentrations grew more slowly than control minnows (Figure 2).

In addition to growth rate, we looked for evidence of developmental (also called fluctuating) asymmetry in response to acute genistein exposure. Asymmetry is cited frequently as an indicator of environmental perturbation, though its utility

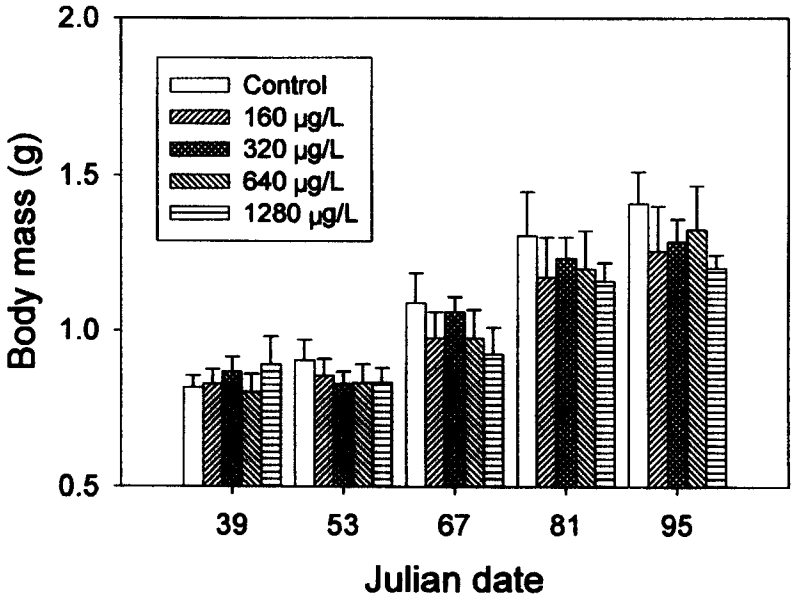


Figure 2. Growth of juvenile fathead minnows over eight weeks following acute exposure to genistein.

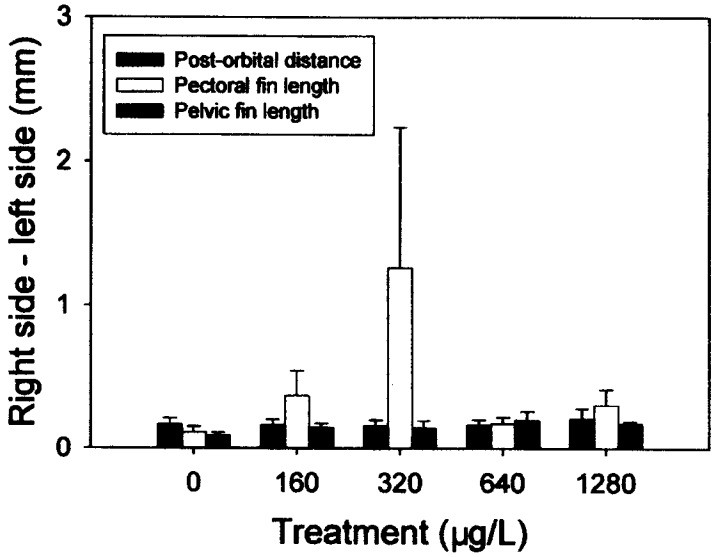
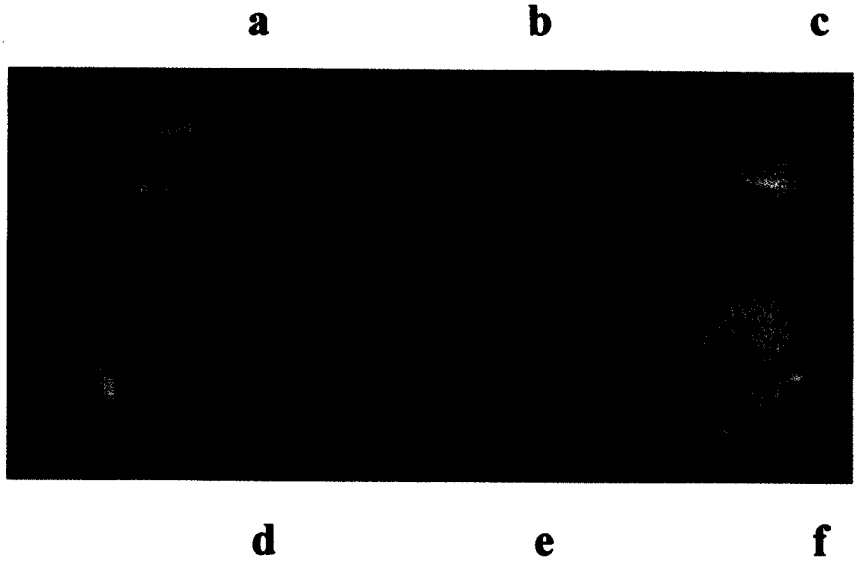


Figure 3. Degree of bilateral asymmetry exhibited by juvenile fathead minnows eight weeks after acute exposure to genistein.



Figures 4a-f. *Xenopus* embryos 48 hr post-fertilization in (a) control conditions, (b) ethanol only and (c) 80 μg/L, (d) 160 μg/L, (e) 320 μg/L, or (f) 1280 μg/L genistein dissolved in ethanol.

has been debated (Leung et al. 2000). We compared left and right side differences in three paired, morphologically independent traits: the post-orbital distance and the lengths of the pectoral and pelvic fins. Genistein exposure did not increase asymmetry in any of the three traits ($P > 0.13$ for all traits; Figure 3). Body size was not a significant covariate in these analyses. The asymmetry in pectoral fin length in minnows exposed to 320 μg/L, while not statistically significant, may indicate a nonlinear dose-response relationship between genistein and trait asymmetry, but this relationship merits further investigation.

Genistein did, however, have a dramatic effect on *Xenopus* embryonic development (Figures 4a-f), which is consistent with its known interference with epidermal growth factor receptors (Akiyama et al. 1987). In the 80 μg/L treatment development was arrested at DAI stage 4. DAI 5 represents a normally developing embryo; scores of 4 or less indicate that the embryo is increasingly posteriorized. Embryos in the 160 and 320 μg/L treatments terminated development at DAI 3 and 2, respectively. Embryos in the 1280 μg/L treatment failed to gastrulate at all within the 48-hr period. Pigmentation differences between treated (Figures 4c-e) and control embryos (Figures 4a, b) are consistent with a recent report by Bevan et al. (2003) that environmental estrogens such as nonylphenol and methoxychlor can alter the deposition of melanocytes in *Xenopus laevis* embryos. There were no differences between the control treatments with or without ethanol, suggesting that the vehicle had no negative effect on development.

The results from our *Xenopus* experiment suggest that, along with reproductive impairment, disruption of embryonic development may be a significant problem for aquatic vertebrates living downstream from pulp and paper mills. Future studies on the role of genistein as an environmental contaminant should focus on three important areas. First, more analytical work in the field must be done to determine what concentrations of phytoestrogens aquatic animals are typically exposed to. Second, more attention needs to be paid to basic population parameters such as age at maturity, spawning success, and juvenile recruitment. Finally, laboratory studies should focus on testing the effects of chemical mixtures similar to those released by pulp and paper mills to better replicate the conditions that animals are likely to experience in the field.

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