

The novel application of an immunological technique reveals the immunosuppressive effect of phytoestrogens in *Betta splendens*

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(Received 10 May 2005, Accepted 14 September 2005)

The novel application of a standard technique to assess cell-mediated immune response to phytohaemagglutinin (PHA) injected in the caudal peduncle in a small fish (<2 g) to test the immunosuppressive effect of three phytoestrogens: genistein, equol and β -sitosterol is described. Individual *Betta splendens* exposed to these oestrogenic chemicals produced weaker inflammatory responses to PHA than did control individuals, suggesting that phytoestrogens are immunosuppressive. This technique should enable immune function in fish species, too small to provide sufficient blood for conventional immunological measures, to be assessed.

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Keywords: *Betta*; cell-mediated immune response; immunosuppression; phytoestrogens; phytohaemagglutinin.

Assessments of immune function in fishes have played an important role in studies examining population biology (Karrow *et al.*, 1999; Cooper, 2002; Scharsack *et al.*, 2004), toxicology (Heath, 1995; Arkoosh *et al.*, 1998), sex differences (Vainikka *et al.*, 2004) and life-history evolution (Milston *et al.*, 2003). Conventional assessments of fish immunity typically include measures of lymphocyte proliferation, lysozyme and anti-protease activity and antibody production (Stolen *et al.*, 1990). These techniques, however, often require volumes of blood (>50 μ l) that preclude their usage with small-bodied fishes. As part of a larger research programme examining the effects of endocrine disrupting chemicals on fish behaviour, a technique was sought to measure a component of immune function suitable for a small tropical fish, *Betta splendens* Regan.

This paper reports the application of a simple immunological test to measure mitogenic T-cell proliferation response to phytohaemagglutinin (PHA), a lectin derived from red kidney beans. PHA stimulates a cell-mediated immune

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response characterized by an inflammatory response where lymphocytes and macrophages infiltrate the site, causing localized swelling (Stadecker *et al.*, 1977; Goto *et al.*, 1978); PHA response has been linked with parasite resistance in poultry (Bayyari *et al.*, 1997). Exposure to PHA provides a nonspecific mitogenic assessment of the cellular immune response and has served as an effective measure for immune function in captive and wild vertebrates (Sheldon & Verhulst, 1996; Klasing, 1998; Svensson *et al.*, 2001; Davison, 2003; Derting & Compton, 2003; Møller & Saino, 2004) and as a tool in ecotoxicology (Smits & Williams, 1999; Grasman, 2002)

Phytoestrogens, which include the isoflavones, coumestans and lignans, are plant compounds structurally similar to animal oestrogens. Phytoestrogens are important components of both human and laboratory animal diets, and thus significant attention has been paid to their health-related effects. The isoflavone genistein, for example, is known to bind to the mammalian oestrogen receptor ER α and induces myriad physiological changes in mammalian models, including steroidogenesis and cell proliferation (Whitten & Patisaul, 2001). Included among these changes in mammals are immunosuppressive effects such as decreased thymus mass, reduced T-cell abundance and reduced cell-mediated immune function (Yellayi *et al.*, 2002; 2003).

There has been a smaller, parallel, interest in the role of phytoestrogens as environmental contaminants. Effluent from pulp and paper mills contains biologically relevant levels of isoflavones and plant sterols (MacLatchy & Van Der Kraak, 1995; Kiparissis *et al.*, 2001). Isoflavonoid phytoestrogens have also been identified in sewage treatment plant effluent and runoff from agricultural fields (Spengler *et al.*, 2001; Kiparissis *et al.*, 2003). Although the consequences of phytoestrogen exposure for wild fish populations are unknown, laboratory studies show significantly reduced steroidogenesis, increased gonadal intersex and developmental abnormalities (Kiparissis *et al.*, 2003; Leusch & MacLatchy, 2003; Ingham *et al.*, 2004), however, no study has specifically examined the effect of phytoestrogens on immune function in fishes.

Whether exposure to PHA would stimulate an immune response was tested by comparing the swelling response of PHA-exposed fish to those injected with phosphate buffered saline (PBS). Domestic male *B. splendens* were obtained from a commercial supplier and housed individually in 1 l beakers in reconstituted reverse-osmosis water maintained at 27° C on a 12 L : 12 D cycle. Fish were fed a daily ration equivalent to 4–5% of their body mass. Each individual was anaesthetized with tricaine methanesulphonate (Western Chemical, Ferndale, WA, U.S.A.; 886-86-2), weighed on a digital balance (± 0.1 g accuracy) and placed on a wet sponge under a $\times 6.3$ dissecting microscope. In the caudal peduncle (*c.* 2 mm from the base of the caudal fin) three to five scales were removed to mark the injection site for consistent measurements; the caudal peduncle was chosen because it is a discrete location amenable to repeatable measurements of swelling. Prior to injection, the thickness of the body at the location of scale removal was measured with a digital micrometer (± 0.001 mm accuracy) three times (repeatability $F_{48,98}$, $P < 0.001$, repeatability = 0.97). After measurements, each individual was injected at the location of scale removal with 4 μ g of phytohaemagglutinin (PHA, Sigma-Aldrich, L-8751) in

2 µl of phosphate buffered saline (PBS) using a 10 µl 26-gauge Hamilton syringe (Hamilton Company, Reno, NV, U.S.A.; 80300). The proper dosage to maintain a similar ratio of body size to PHA volume (1–2 µl per g body mass) used in avian studies dosage was determined. After 24 h, each fish was anaesthetized again and the thickness of the tissue at the location of injection and scale removal was remeasured (post-injection $F_{48,98}$, $P < 0.001$, repeatability = 0.82). The response of each individual was recorded as the ratio of post-injection thickness to pre-injection thickness (Smits *et al.*, 1999). Exposure to PHA induced an inflammatory response, while exposure to PBS only did not (mean ± s.e.: PBS 1.05 ± 0.10 , $n = 5$; PHA + PBS 1.41 ± 0.13 , $n = 5$; Mann–Whitney $U = 1.00$, $P = 0.01$), suggesting that injection with PHA stimulated a cell-mediated immune response. Certainly, any test that punctures a fish's integument can lead to infection and thus may influence the very measure under consideration; however, no evidence of infection was found, even weeks after exposure. There was no evidence that injections *per se* caused localized swelling in *B. splendens* (one-sample *t*-test PBS *v.* mean = 1.0, d.f. = 4, $P > 0.05$). Rather, individuals injected with PHA mounted a stronger inflammatory response than those exposed to PBS alone. In addition, the injection of the PHA should cause no greater (and perhaps less given the small gauge of the needle) penetration of the skin barrier than many standard blood collecting techniques.

Whether exposure to three phytoestrogens (genistein, equol and β-sitosterol) would affect immune responses to PHA was then tested. Each fish was randomly assigned to one of the following four treatments: control, genistein (1000 µg l⁻¹; Sigma-Aldrich, St Louis, U.S.A.; G6649), equol (1000 µg l⁻¹; Apin, Abingdon, U.K.; N04392) and β-sitosterol (1000 µg l⁻¹; Sigma-Aldrich; S1270). These levels are at the upper limit of those reported in nature (MacLatchy & Van Der Kraak, 1995), though are well within the sublethal range for this species (E.D. Clotfelter & A. Rodriguez, unpubl. data). Phytoestrogens were dissolved in an ethanol vehicle and control fish were exposed to similar ethanol fractions mixed with tank water. The exposure period was 28 days and fish were injected with PHA 11–15 days following the end of the exposure period. The latter time period was used in order to accommodate a schedule of behavioural observations performed on these fish as part of an unrelated study; phytoestrogens are known to have transgenerational effects in fishes (Lehtinen *et al.*, 1999; Nakari & Erkomaa, 2003), thus the effects of this delay are negligible. Ten percent (100 ml) of the water volume was replaced (with water of the same nominal concentration) on alternating days. Because data were not normally distributed (Shapiro–Wilk's W , $P < 0.01$), data were log₁₀-transformed to achieve normality (Shapiro–Wilk's W , $P > 0.05$). The possible immunosuppressive effect of phytoestrogens was tested using a two-factor ANOVA (SAS, 1988) that included body mass as a covariate to control for differences in size among individuals; fish did not lose body mass over the measurement period ($P = 0.54$). Exposure to phytoestrogens reduced immune response in all treatment groups ($F_{3,34}$, $P = 0.003$; Fig. 1), but there was no effect of body mass ($F_{1,34}$, $P = 0.46$).

These results demonstrate that researchers interested in behaviour, sexual selection and life-history evolution in small fishes can easily measure

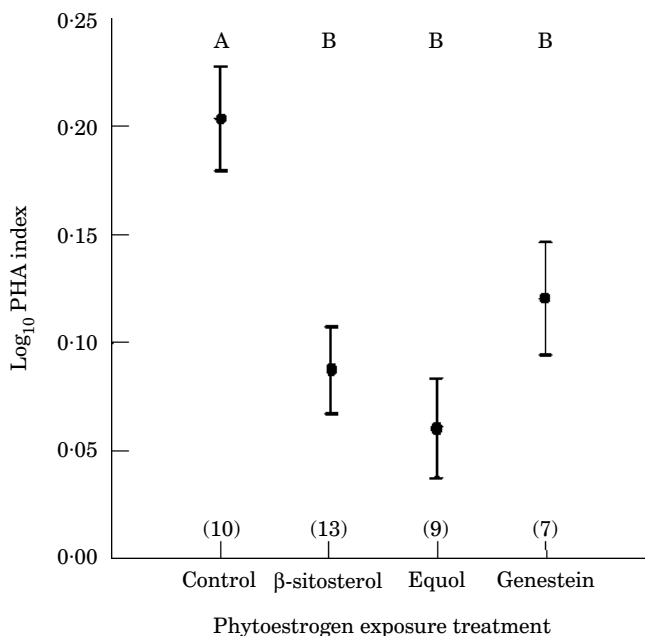


FIG. 1. The effect of exposure to three phytoestrogens on cell-mediated immune response to phytohaemagglutinin (PHA). PHA response is reported as least square means \pm s.e. of the \log_{10} of the ratio of post-injection thickness to pre-injection thickness. Letters above means refer to significant differences in values; sample sizes are given in parentheses.

cell-mediated immune function. Such an approach has been used widely in birds and other vertebrates (Sheldon & Verhulst, 1996), but has seen no application to fishes. This simple skin test is not intended to replace more sophisticated measures of immune function and should provide a more quantitative alternative than allograft studies (Grether *et al.*, 2004). Rather, for studies on small fishes for which the collection of large quantities of blood is not feasible, this test can provide a way to assess immune function.

With respect to the immunosuppressive effects of phytoestrogens on fishes, these results are consistent with those reported for rodents by Yellayi *et al.* (2002, 2003) and add to the growing literature suggesting that these chemicals are significant environmental contaminants. The fact that similar effects of genistein, equol and β -sitosterol were observed suggests that vertebrate populations in the vicinity of sewage treatment plants, pulp mills and agricultural feedlots are all potentially at risk. While the concentrations tested are higher than those reported *in situ* (Kiparissis *et al.*, 2001), these results should encourage ecotoxicologists to use this and other techniques to examine immunosuppression in fishes as a consequence of phytoestrogen exposure.

We thank M. Manning and A. Rodriguez for their assistance with all aspects of fish maintenance associated with this study and E. Rice for comments on an earlier version of this manuscript. This work was done with approval of the Amherst College Institutional Animal Care and Use Committee and with funding from the Dean of Faculty's office at Amherst College.

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