Effects of the Isoflavones Genistein and Equol on the Gonadal Development of Japanese Medaka (*Oryzias latipes*)

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The estrogenic isoflavone compound genistein recently has been found in the effluents of sewage treatment plants and pulp mills, and the related compound equol has been detected in the runoff from agricultural fields treated with hog manure. Waterborne exposures of Japanese medaka (*Oryzias latipes*) to equol from soon after hatch to approximately 100 days posthatch induced gonadal intersex (i.e., testis-ova) in males at incidences of 10 and 87% in equol treatments of 0.4 and 0.8 µg/L, respectively. Exposure to the highest test concentration of genistein, 1,000 µg/L, also caused a low incidence (i.e., 12%) of gonadal intersex in male medaka. The ovaries of female medaka from both equol and genistein treatments showed delayed oocyte maturation, atretic oocytes, an enlarged ovarian lumen, proliferation of somatic stromal tissue, and primordial germ cells; responses were concentration dependent. Alterations to externally visible secondary sex characteristics occurred in medaka exposed to both equol and genistein. In treatments with 1,000 µg/L genistein, 72% of male medaka (as identified by the gonadal phenotype) showed feminized secondary sex characteristics. Gonadal intersex and alterations to secondary sex characteristics have been noted in several fish populations around the world. This laboratory study indicates that isoflavone compounds should be considered candidate estrogenic compounds that may be involved in the alteration of sexual development in feral fish populations. *Key words:* endocrine disruption, equol, genistein, intersex, isoflavones, Japanese medaka. *Environ Health Perspect* 111:1158–1163 (2003). doi:10.1289/ehp.5928 available via *http://dx.doi.org/* [Online 5 February 2003]

Evidence shows that endocrine-disrupting chemicals (EDCs) can alter gonadal development and sexual differentiation in populations of wild fish. For instance, EDCs present in the effluent of a Swedish pulp mill affected sexual differentiation in eelpout (*Zoarces viviparous*), as indicated by sex ratios dominated by the male phenotype (Larsson et al. 2000). In roach (*Rutilus rutilus*) and gudgeon (*Gobio gobio*) populations from rivers in the United Kingdom, there was a high prevalence of gonadal intersex, which was attributed to EDCs discharged from the effluents of sewage treatment plants (STPs) (Jobling et al. 1998, 2002; Van Aerle et al. 2001). Intersex gonads were also present in male flounder (*Platichthys flesus*) in a contaminated estuary in the United Kingdom (Allen et al. 1999; Simpson et al. 2000), in barbel (*Barbus plebejus*) from a polluted tributary in Italy (Viganò et al. 2001), in shovelnose sturgeon (*Scaphirhynchus platyorynchus*) from the Mississippi River near the sewage discharges of St. Louis, Missouri, USA (Harshbarger et al. 2000), and in white perch (*Morone americana*) from the lower Great Lakes region (Kavanagh et al. 2002). Bleached kraft mill effluents (BKME) can also affect the expression of secondary sex characteristics in fish. BKME exposure caused masculinization of female mosquitofish (*Gambusia affinis*), indicated by the development of malelike gonopodia (anal fin) and reproductive behavior (Bortone et al. 1989; Cody and Bortone 1997).

Because the effluents from industrial, domestic, and agricultural sources are complex mixtures of chemicals, the specific EDCs responsible for effects on gonadal development and sexual differentiation in fish have not been identified conclusively (Sumpter 1998). Possible EDCs involved in the disruption of gonadal development in fish include endogenous and synthetic sex steroids, alkylphenol surfactants, and the plasticizer bisphenol A (Jenkins et al. 2001; Kolpin et al. 2002; Metcalfe et al. 2001; Sheahan et al. 2002; Spengler et al. 2001; Ternes et al. 1999), pesticides (Harshbarger et al. 2000), and phytosterols such as β-sitosterol (MacLatchy and Van Der Kraak 1995; Mellanen et al. 1996).

Recent studies have shown that isoflavone compounds, including genistein and equol (Figure 1), are discharged in effluents from STPs and from pulp mills and are present in agricultural runoff from intensive livestock operations. For instance, genistein was isolated and identified at a concentration of 10 µg/L in the final BKME from a pulp mill in Ontario, Canada (Kiparissis et al. 2001). Genistein was also present at concentrations ranging from 3 to 38 ng/L in 60% of the STP effluents sampled in Germany by Spengler et al. (2001). Equol, produced as a metabolite of formonetin and daidzein in the gut microflora of farm animals, was present at 10–1,300 ng/L concentrations in the runoff from a field amended with hog manure (Burnison et al. 2000). In addition, farmed fish can be exposed to equol and genistein through dietary sources because the vegetable components of fish food often have high levels (> 30%) of soya and alfalfa—plant sources rich in isoflavonoids (Pelissero and Sumpter 1992).

Mammalian studies have demonstrated the endocrine-modulating potential of isoflavones. In Australia during the 1940s, ewes that foraged on clover experienced temporary or permanent infertility (Harborne and Williams 2000). Laboratory studies with mammalian models have shown that genistein and equol are estrogen agonists (Birt et al. 2001). There have been very few studies on the effects of genistein and equol in teleosts. Intraperitoneal (ip) injections of genistein and equol to yearling Siberian sturgeon (*Acipenser baeri*) induced vitellogenesis, a physiological response associated with estrogenic activity (Pelissero et al. 1991). In Japanese medaka (*Oryzias latipes*) exposed intraperitoneally to genistein, vitellogenin was not induced in males and females, but plasma 17β-estradiol (E_2) was increased in exposed females and plasma testosterone (T) levels were reduced in exposed males (Zhang et al. 2002). Genistein-enriched diets were fed to rainbow trout (*Oncorhynchus mykiss*) for 1 year, and gametogenesis and reproductive efficiency of the trout were reduced (Bennetau-Pelissero et al. 2001).

Although the studies described above demonstrated that equol and genistein interfere with endocrine and reproductive processes in teleosts, no studies have been conducted to determine the effects of these compounds on the gonadal development of fish. With this study we attempted to evaluate the impacts of waterborne exposures to equol and genistein on the development and differentiation of the gonad and the expression of secondary sex characteristics in fish. We used the Japanese medaka, an aquarium oviparous fish, as a laboratory model species, because its developmental history has been well characterized and it is sensitive to effects on gonadal development when exposed to estrogenic compounds (Cheek et al. 2001; Edmunds et al. 2000; Gray and Metcalfe 1997; Metcalfe et

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al. 1999, 2001; Nimrod and Benson 1998; Yamamoto 1965; Yamamoto and Matsuda 1963). Spawning in medaka can be induced multiple times throughout the year by manipulating only the temperature and photoperiod, which makes this fish species suitable for studies of reproduction and gonadal development (Metcalfe et al. 1999).

Materials and Methods

We conducted the Japanese medaka reproductive development assay according to an existing protocol by Metcalfe et al. (2001). Briefly, we daily collected newly hatched medaka embryos and placed them in glass containers or aquaria of progressively larger sizes (according to medaka growth) containing 1, 2, and 10 L dechlorinated tap water. We prepared stock solutions of genistein and equol by dissolving the isoflavones in acetone (distilled in glass) and administered the stock solutions into the static exposure systems at a ratio of 100 µL of stock solution per liter of water. We added the same amounts of acetone to test containers in the control treatments. The nominal concentrations for genistein (Sigma-Aldrich, Toronto, Ontario, Canada) were 1, 10, 100, and 1,000 µg/L, and for equol (Apin Chemicals, Oxfordshire, England) the nominal concentrations were 0.4 and 0.8 µg/L. To select the highest nominal exposure concentrations, we relied on determining the no-observed-adverse-effect concentration in preliminary embryotoxicity tests with early life stages of medaka, which we carried out according to Harris et al. (1994). We changed 100% of the water in the test containers to renew the exposure concentrations three times per week during the exposure period. We maintained medaka under a 16-hr light:8-hr dark cycle and a water temperature of 25 ± 2°C. We fed the fish *ad libitum* with a diet of newly hatched San Francisco Bay brine shrimp at a rate of three times a day throughout the exposure period.

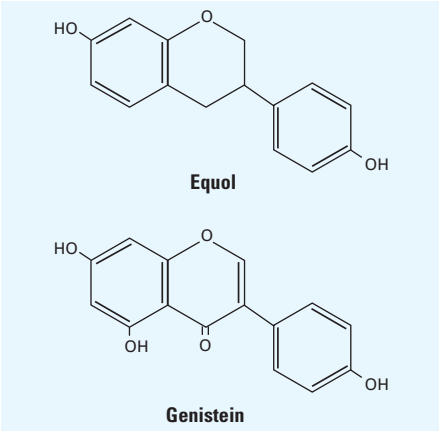

Figure 1. Molecular structure of the isoflavone compounds genistein and equol.

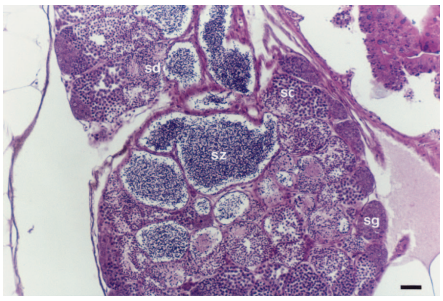

Figure 2. Normal testis at the intermediate stage of development. All stages of spermatogenesis are present with the immature spermatogonia (sg) and spermatocytes (sc) found in the periphery and the mature spermatids (sd) and spermatozoa (sz) in the middle of the testicular tissue (H&E, 150×). Bar = 10 µm.

When medaka reached reproductive maturity approximately 100 days after hatch, we euthanized them with an overdose of tricaine methane sulfonate (MS-222; Fisher Scientific, Toronto, Ontario, Canada). Under a dissecting microscope, we assessed the phenotypic sex of individual medaka by examining externally visible secondary sex characteristics, including the shape of the urogenital papilla, the shape of the dorsal fin, and the shape and the presence or absence of papillary processes on the anal fin. We then placed the fish in tissue capsules and fixed them in Calex fixative (Fisher Scientific). We dehydrated all medaka histological samples in a graded alcohol series and embedded them in paraffin. From each medaka we took sagittal sections (5–7 µm) in a step-section manner (12–20 sections per fish), and we mounted them on microscope slides and stained them with hematoxylin and eosin (H&E), using standard staining techniques.

We evaluated the type and condition of the gonadal tissue and the various stages of gametogenesis of all individual fish under a compound microscope. We classified the stages of oogenesis in individual female medaka as previtellogenic (pre-VtG), VtG, and post-VtG (or postovulatory) according to criteria developed by Iwamatsu et al. (1988). For the stages of spermatogenesis, we primarily used the criteria developed by Grier (1981), which we modified according to personal observations. Briefly, during the immature stages, testicular cysts are filled primarily with spermatogonia and primary spermatocytes, and in the intermediate stages, the cysts are filled with all types of sperm cells (Figure 2), whereas in the advance stages, cysts are filled primarily with mature spermatozoa.

In general, we considered that medaka with total body lengths greater than 17 mm have passed the immature stages of gonadal development and have developed well-defined secondary sex characteristics. Therefore, we included only individual medaka that met this prerequisite body length in our statistical analyses of the development of the gonad and secondary sex characteristics. We used χ^2 analysis (SigmaStat for Windows, Version 1, 1992–1994; Jandel, San Rafael, CA, USA) to evaluate the effects of equol and genistein on gonadal development, gametogenesis, and expression of secondary sex characteristics.

Results

Equol treatments. The mortality rate of medaka in all equol treatments was similar to that of the control group (data not shown). The ratios of female and male medaka in the equol treatments were not statistically different from the ratios in the control treatment (Table 1). Exposure to 0.4 and 0.8 µg/L equol resulted in the development of intersex (i.e., testis-ova) in 2 (10%) and 13 (87%) of the males, respectively (Table 1). In all cases of testis-ova, oogonia were present throughout the testicular tissue (Figure 3). In the control treatment, the process of spermatogenesis was advanced, whereas in the equol treatments, spermatogenesis was relatively retarded in a concentration-dependent manner (χ^2 analysis; $p < 0.05$). Approximately 92% of the male medaka were in the advanced stages of spermatogenesis in the control treatment, compared with 38 and 7% in the 0.4 and 0.8 µg/L treatments, respectively (Table 2). We observed proliferation of fibrotic tissue and a decrease in the density of spermatozoa in the testis of four males (19%) and six males (29%) in the 0.4 and 0.8 µg/L treatments, respectively (Table 2), which was concentration dependent (χ^2 analysis; $p < 0.05$).

Exposure to equol also had an effect on oogenesis in the female medaka that met criteria for examination (i.e., > 17 mm long).

Table 1. Number (percent) of female and male medaka (*O. latipes*) histologically identified, and number (percent) of phenotypic male medaka with the intersex condition testis-ova.

Chemical, nominal concentration (µg/L)	Total no.	Females	Males	Testis-ova
Genistein				
0	61	34 (56)	27 (44)	0
1	64	34 (53)	30 (47)	0
10	65	43 (66)	22 (34)	0
100	57	35 (61)	22 (39)	0
1,000	48	31 (65)	17 (35)	2 (12)
Equol				
0	27	14 (52)	13 (48)	0
0.4	46	25 (54)	21 (46)	2 (10)
0.8	43	28 (65)	15 (35)	13 (87)

For instance, in the 0.4 µg/L treatment, 80% of the females had VtG oocytes and 20% were at the postovulatory or spawning stage. In females exposed to 0.8 µg/L equol, significantly fewer females (11%) were in spawning condition (χ^2 analysis; $p < 0.05$). In the control treatment, 57% of females were at the VtG stages of ovarian development (Figure 4) and 43% were in spawning condition (Table 3). Other characteristics of ovarian development that we evaluated included the presence of atretic oocytes (Figures 5 and 6), the presence of a large ovarian lumen (OL) (Figure 6), and the development of somatic stromal tissue in the ovary (Figure 5). The prevalence of oocyte atresia, a large OL, and somatic stromal tissue were 40, 15, and 15%, respectively, in the 0.4 µg/L treatment, and 25, 25, and 54%, respectively, in the 0.8 µg/L treatment. We also observed atresia and a large OL in females from the control treatment, but at a much lower incidence, and we did not observe any stromal tissue in control females (Table 3). Primordial germ cells (PGCs) were also visible in the ovaries of female medaka exposed to 0.8 µg/L equol (Figure 7) at an incidence of 18%. PGCs were not present in the ovaries of females from the 0.4 µg/L equol or control treatments (Table 3).

Congruence between secondary sex characteristics and gonadal sex declined in a concentration-dependent manner in the male and female medaka (Figure 8). In the control treatment, we erroneously identified only one medaka as a male from the appearance of the dorsal and anal fins, whereas histology of the gonad revealed it to be a female. In the treatment with 0.4 µg/L equol, we observed five females (20%) that had male-like dorsal fins and anal fins with the notch characteristic of males. In the same treatment, there were six males (29%) in which the dorsal and anal fins as well as the urogenital pore were characteristic of the female phenotype. In the treatment with 0.8 µg/L equol, 57% of females and 73% of males had secondary sex characteristics showing the opposite phenotype from the gonadal sex.

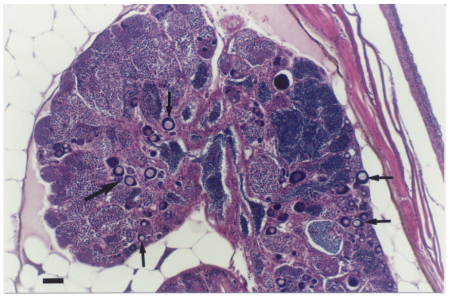

Figure 3. Testis-ova observed in a Japanese medaka (*O. latipes*) exposed to 0.8 µg/L equol. Immature pre-VtG oocytes (arrows) are distributed throughout the testicular tissue (H&E, 100×). Bar = 20 µm.

Genistein treatments. Genistein was not lethal to Japanese medaka in all test concentrations (data not shown). The ratios of female and male medaka in the genistein treatments were not statistically different from the ratios in the control treatment (Table 1). We observed testis-ova in the gonads of only two fish exposed to the highest nominal concentration of genistein (1,000 µg/L; Table 1). There appeared to be no significant effects of genistein exposure on spermatogenesis in male medaka. However, other characteristics of testicular development were altered in genistein treatments. There was a significant concentration-dependent increase (χ^2 analysis; $p < 0.05$) in the numbers of male fish with connective tissue in the testis and fibrosis around the testicular lobules, as well as an increase in the numbers of fish showing low densities of spermatozoa in both the testicular lobules and efferent ducts (Table 2).

Among female medaka, the stages of oogenesis showed concentration-dependent changes (χ^2 analysis; $p < 0.05$). As shown in Table 3, among females in the 10 µg/L treatment, 47% of medaka chosen for examination (> 17 mm long) had oocytes at the pre-VtG stage of development and 53% had oocytes at the early VtG stages of oogenesis, with only one female being at the late VtG stage (postovulatory). In comparison, in the control treatment, oogenesis was more advanced, as indicated by the presence of VtG oocytes in 91% of female medaka (Table 3). The incidence of oocyte atresia increased in female medaka in a concentration-dependent manner from 9 to 38% in the genistein treatments. There was also a concentration-dependent increase in the incidence of fish with reduced numbers of oocytes and a consequent increase in the area of the OL from 10 to 27% in the genistein treatments. Finally, the incidence of fish with ovaries in which PGCs were visible increased from 5% in the 1 µg/L treatment to 19% at the highest concentration (Table 3).

In the medaka from the control treatment, the secondary sex characteristics were in agreement with the gonadal sex in 96% of females and 100% of males (Figure 6). In medaka identified as females from the appearance of the gonad, the agreement between gonadal sex and secondary sex characteristics in the various genistein treatments ranged from 56 to 61% (Figure 8), which was statistically different from controls ($p < 0.05$). In medaka identified as males from the appearance of the gonad, there was poor agreement between external characteristics and gonadal sex (38%) in the highest genistein concentration (Figure 8). The shape of the urogenital pore was altered to the female phenotype (i.e., feminized) in all of the affected male medaka.

Discussion

In tests with Japanese medaka laboratory models, genistein and equol altered the development of the gonad and secondary sex characteristics. The most well-defined developmental response observed in both isoflavone treatments was the induction of gonadal intersex, as characterized by the presence of testis-ova. Equol caused a concentration-dependent increase in the incidence of testis-ova, but genistein only induced a low incidence of testis-ova at the highest test concentration. Exposure to known xenoestrogens and endogenous and synthetic

Table 2. Histological determination of stages of spermatogenesis and gonadal effects in phenotypic male medaka[a] (*O. latipes*) exposed to various nominal concentrations of equol and genistein.

		Stages of spermatogenesis, no. (%)			Gonadal effects, no. (%)		
Chemical (µg/L)	Total no.	Immature	Intermediate	Advanced	Normal	Fibrosis	Spermatozoa[b]
Genistein							
0	17	3 (18)	5 (29)	9 (53)	17 (100)	–	–
1	18	7 (39)	7 (39)	4 (22)	11 (61)*	5 (28)	2 (11)
10	10	1 (10)	7 (70)	2 (20)	4 (40)*	4 (40)	4 (40)[c]
100	13	2 (15)	6 (46)	5 (38)	4 (31)*	4 (31)	6 (46)[c]
1000	6	3 (50)	3 (50)	–	1 (17)*	3 (50)	3 (50)[c]
Equol							
0	13	–	1 (8)	12 (92)	13 (100)	–	–
0.4	21	–	13 (62)	8 (38)*	9 (43)*	4 (19)	6 (29)[d]
0.8	15	–	14 (93)	1 (7)*	2 (13)*[d]		

[a]Male medaka with total length > 17 mm were used for gonadal evaluation. [b]Low density of mature spermatozoa. [c]Some of the medaka showed both gonadal defects. [d]Gonads of 9% (0.4 µg/L) and 87% (0.8 µg/L) of medaka developed testis-ova. *Statistical difference between individual treatments and their corresponding control groups at $p < 0.05$.

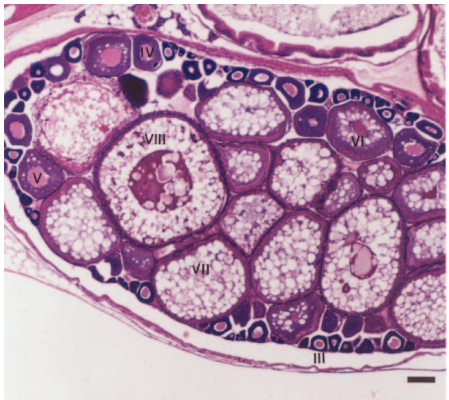

Figure 4. Normal ovary at late VtG stages of development. Pre-VtG oocytes (III and IV) are mainly located in the periphery, whereas VtG oocytes (V–VIII) are located in the middle of the ovarian tissue (H&E, 60×). Bar = 20 µm.

steroidal estrogens induces testis-ova in male medaka, as indicated by the presence of pre-VtG oocytes within the testis (Gray and Metcalfe 1997; Metcalfe et al. 1999, 2001; Wester and Canton 1986). In Japanese medaka, a true differentiated gonochoristic species, spontaneous sex reversal, or intersex, has never been observed (Yamamoto 1958). This is the first study to show that exposure of fish to isoflavone compounds can induce gonadal intersex. In addition, intersex was induced at nominal concentrations that are ecotoxicologically relevant (i.e., micrograms per liter). However, the concentrations of the test compounds were not monitored throughout the exposure period. It is possible that the exposure concentrations in the static renewal test system were lower than the nominal concentrations reported here. Future studies should determine the effects in fish in relation to measured exposure concentrations.

Intersex gonads in male medaka exposed to genistein and equol are an indication of their estrogenic activity. Both genistein and equol give a positive response in the yeast estrogenicity assay, with potencies relative to E_2 of 2×10^{-3} and 3×10^{-3}, respectively (Kiparissis 2001). Although the *in vitro* estrogenic potencies of these compounds are similar, equol showed a higher estrogenic potency in the *in vivo* assay with medaka, as indicated by high rates of induction of testis-ova at concentrations < 1 µg/L. Elsewhere, dietary exposure of genistein caused an elevation of plasma vitellogenin levels in Siberian sturgeon, but not in rainbow trout, indicating interspecies variability (Latonnelle et al. 2002). Other factors, such as the relative water solubilities of the test compounds in the exposure vessels, the relative rates of bioaccumulation of genistein and equol by exposed medaka, and the relative rates of metabolism, are important factors in these *in vivo* tests. Sex-related differences regarding the uptake and metabolism of genistein have been described in mammals (Coldham and Sauer 2000) and may also apply to teleosts. Therefore, caution must be taken in relating the *in vitro* potencies of genistein and equol to the relative potencies for *in vivo* responses in fish and other vertebrates.

Apart from induction of testis-ova in males, equol and genistein induced other effects on the testis of exposed male medaka. Spermatogenesis was inhibited in males exposed to both equol treatments. On the other hand, spermatogenesis was only slightly altered in medaka exposed to genistein. In another study, male rainbow trout fed a diet consisting of 500 and 1,000 ppm genistein experienced an apparent acceleration of spermatogenesis, which produced a dose-dependent decrease in sperm density and motility (Bennetau-Pelissero et al. 2001). A decrease in the density of mature sperm cells in male medaka was observed in our study in all equol and genistein treatments. There was also a significant increase in the amount of fibrotic tissue around the testicular lobules in some medaka exposed to equol and genistein. However, the severity of this response is not easily quantifiable. It was suggested that testicular fibrosis and a concurrent reduction in the concentration of spermatozoa are the prerequisites for development of oocytes in the intersex testis (Egami 1955; Konno and Egami 1966). Exposure to E_2, 17α-ethinylestradiol (EE_2), and estriol at concentrations ranging from 10 to 1,000 ng/L caused similar effects (Metcalfe et al. 2001).

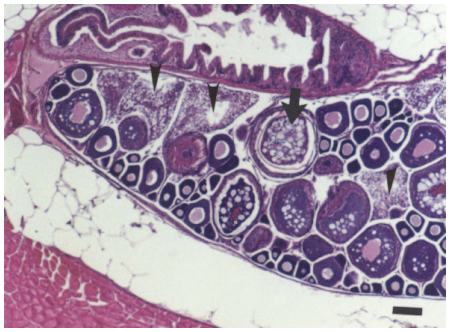

Figure 5. Ovary of a female Japanese medaka (*O. latipes*) exposed to 0.8 µg/L equol, showing atretic oocytes (arrows) and an increase in somatic stromal tissue (arrowheads) (H&E, 40×). Bar = 50 µm.

Testicular fibrosis was also present in sheepshead minnows (*Cyprinodon variegatus*) exposed to 200–800 ng/L EE_2 (Zillioux et al. 2001), indicating that this response is widespread in fish species exposed to potent estrogens.

Both genistein and equol also altered development of the ovary and oogenesis in female medaka. In general, the ovary of the Japanese medaka is an unpaired saclike organ attached to the wall of the coelomic cavity. It is lined with epithelium cells and within the epithelium there is a layer of mesenchymal connective tissues. From the ovarian wall, numerous folds project toward the middle of the ovary that comprise oocytes at various stages of development, attached to a loosely

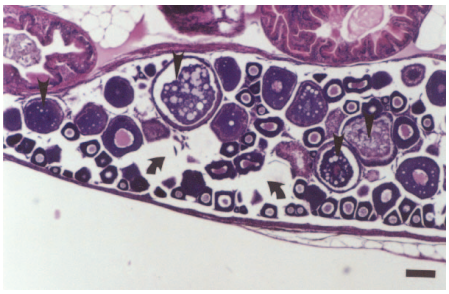

Figure 6. Ovary of a female Japanese medaka (*O. latipes*) exposed to 0.8 µg/L equol, showing an enlarged OL (arrows). Atretic oocytes are also indicated with arrowheads (H&E, 40×). Bar = 50 µm.

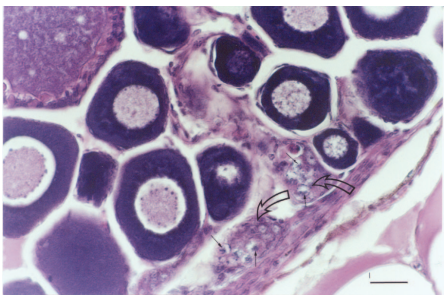

Figure 7. Ovary of a female Japanese medaka (*O. latipes*) exposed to 0.8 µg/L equol, showing PGCs distributed in clusters (large arrows) in the periphery of the ovarian tissue. PGCs (small arrows) are larger than the somatic cells and are characterized by a distinct outline, a less-stained cytoplasm, and a large and roughly spherical nucleus (H&E, 400×). Bar = 10 µm.

Table 3. Histological determination of stages of oogenesis and gonadal effects in phenotypic female medaka[a] (*O. latipes*) exposed to various nominal concentrations of equol and genistein.

Chemical		Stages of oogenesis, no. (%)			Gonadal effects, no. (%)				
(µg/L)	Total no.	Pre-VtG	VtG	Post-VtG	Normal	Atresia	OL	Stroma	PGCs
Genistein									
0	23	2 (9)	21 (91)	–	21 (91)	–	1 (5)	–	1 (4)
1	21	3 (14)	18 (39)	1 (4)	14 (64)	*2 (9)	6 (27)	–	1 (5)[b]
10	17	8 (47)	9 (53)*	–	11 (65)*	3 (18)	3 (17)	–	–
100	20	4 (20)	15 (78)	1 (5)	12 (60)*	4 (20)	2 (10)	–	2 (10)
1,000	21	8 (38)	13 (62)	–	7 (33)*	8 (38)	6 (29)	–	4 (19)[b]
Equol									
0	14	–	8 (57)	6 (43)	10 (71)	3 (21)	2 (14)[b]	–	–
0.4	25	–	20 (80)	5 (20)*	12 (48)*	10 (40)	3 (15)	3 (15)[b]	–
0.8	28	–	25 (89)	3 (11)*	7 (25)*	7 (25)	15 (54)	6 (21)	5 (18)[b]

[a]Female medaka with total length > 17 mm were used for gonadal evaluation. [b]Some of the medaka showed a combination of gonadal defects. *Statistical difference between individual treatments and their corresponding control groups at $p < 0.05$.

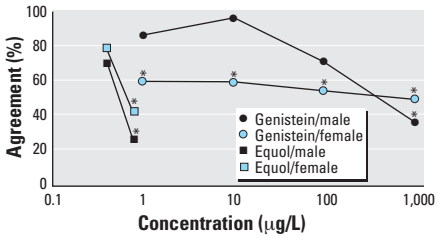

Figure 8. Agreement between the phenotypic gonadal sex and secondary sex characteristics in Japanese medaka (*O. latipes*) exposed to equol and genistein.
*Significant results at $p < 0.05$.

connective tissue or stroma. The OL is located in the dorsal part of the ovary and is connected to the oviduct (Robinson and Rugh 1972). The development of oocytes is asynchronous, with oocytes developing continuously from one stage to the next. Postovulation, many oocytes become atretic, as evident by the deformed shapes and hypertrophied follicular cells of the oocytes. The absorbed atretic oocytes are the precursors of the stromal tissue in the ovary (Yamamoto and Yoshioka 1964). Both isoflavonoids caused a moderate to high incidence of medaka with oocyte atresia. Atretic oocytes are easily recognized in stained tissues by the separation of the oocyte membrane from the ooplasm, or alternatively, separation of the ooplasm from the nucleus. Atresia is part of normal ovarian development at the postovulatory stage. However, it is uncommon in pre-VtG ovaries (Guraya 1986). In addition, equol caused a significant proliferation of intraovarian stromal tissue, even in ovaries with oocytes at the early VtG stage of development. However, an increase in the incidence of fish with reduced numbers of oocytes and a simultaneous increase in the intraovarian space (i.e., OL) were evident in treatments with both isoflavones. Finally, PGCs were present in the ovaries of medaka exposed to both isoflavones. Their presence in the ovaries of mature female medaka is uncommon and may be attributed to inhibition of oocyte growth.

The medaka is a sexually dimorphic species, and mature males are recognizable by external secondary sex characteristics such as the shape of the dorsal fin, where there is a characteristic shallow notch in the posterior end (Egami 1955). Another feature in sexually mature males is the presence of papillary processes on the posterior rays of the anal fin, which are used for mating behavior. Females are recognizable from a distinct round-shaped urogenital papilla, which covers both the genital and urinary pores. Genistein had a pronounced impact on the expression of these secondary sex characteristics. In approximately half the medaka with the female gonadal phenotype in all genistein treatments, the dorsal and/or anal fins were male-like in appearance. In treatments with equol, expression of secondary sex characteristics was altered only in medaka exposed to the highest concentration. The expression of secondary sex characteristics in fish is under steroid hormone control (Brantley et al. 1993). Therefore, alterations in medaka exposed to isoflavones could be attributed to the endocrine-modulating potential of this class of phytochemicals.

Although the estrogenic potential of equol and genistein may explain the testicular alterations and the feminization of secondary sex characteristics in males, especially at the highest test concentrations, it cannot account for the adverse effects on ovarian development and the masculinization of the dorsal and anal fins. In *in vitro* studies with MCF-7 cells, Zava and Duwe (1997) observed that low concentrations of genistein (1 nM–1 μM) caused estrogenic effects, while higher concentrations (> 10 μM) caused antiestrogenic effects. In addition, depending on the levels of the endogenous sex steroid hormones, phytoestrogens such as genistein and equol can act either as estrogen agonists or antagonists (Herman et al. 1995). Bennetau-Pelissero et al. (2001) showed that when plasma E_2 levels were low in immature female rainbow trout, genistein acted as an estrogen agonist, as evident by increased plasma vitellogenin levels. However, during the onset of vitellogenesis when plasma E_2 levels increased, genistein acted as an estrogen antagonist by dramatically delaying the process of oocyte maturation. This effect on oogenesis was also demonstrated in our study in the 10 μg/L genistein treatment and in both equol treatments.

Zava and Duwe (1997) suggested that although genistein is an estrogen agonist, it also cross-talks with other processes that are estrogen-receptor–independent, a hypothesis recognized elsewhere (Cassidy 1998; Diel and Michna 1998). Genistein and equol have the potential to interfere with the function of key enzymes for the biosynthesis and metabolism of estrogens. For example, genistein inhibited the action of 3β-hydrosteroid dehydrogenase Δ_5/Δ_4 isomerase and 17β-hydroxysteroid dehydrogenase in human placental microsomes (Le-Bail et al. 2000). Although genistein and equol inhibited aromatase activity, which catalyzes the conversion of androgens to estrogens, in rainbow trout ovarian microsomes (Pelissero et al. 1996) they were ineffective as inhibitors of aromatase in mammalian tissues (Campbell and Kurzer 1993). Equol can also interfere with fish gonadal development by its ability to competitively bind to the fish androgen receptor (AR) with a potency of 2,200-fold less than testosterone, the natural endogenous ligand (Burnison BK. Personal communication). Genistein failed to bind to rainbow trout hepatic AR (Knudsen and Pottinger 1999). Furthermore, equol and genistein may affect the transport of both androgens and estrogens to target organs by interacting with and modulating the activities of the sex steroid binding proteins (SSBP). In human SSBP, both genistein and equol inhibited the binding of E_2 and testosterone in a dose-dependent manner, but with equol being the more potent inhibitor (Martin et al. 1996). Equol was also 1,000-fold less potent than E_2 in competing for binding sites in fish SSBP (Burnison BK. Personal communication). It is possible that any of the above mechanisms may play a role in the altered ovarian development and masculinization of secondary sex characteristics in female medaka in both genistein and equol treatments.

In field studies, white suckers (*Catostomus commersoni*) exposed to pulp mill effluents experience delays in sexual maturity, lower gonadosomatic indices, reduced numbers of viable eggs, and alterations of secondary sex characteristics (McMaster et al. 1991). In the present laboratory study, both isoflavonoids caused some of these effects. Because of the small size of medaka and their gonadal tissue, data on gonadosomatic indices were not available in our study. However, in the ovaries of some female medaka exposed to both isoflavone compounds, there was a significant increase in the incidence of fish with a large OL (i.e., reduction in number of oocytes), which indirectly indicates a reduction in ovarian weight and consequently a lower gonadosomatic index. The most pronounced effect on ovarian development observed in most of the treatments with isoflavones was the reduction in the number of mature oocytes in the ovarian cavity, which is possibly linked to high rates of oocytic atresia. Feral fish exposed to pulp mill effluents also experienced a high incidence of ovarian atresia (Janz et al. 1997). Another significant effect, especially for medaka exposed to genistein, was the alteration of the phenotypic expression of secondary sex characteristics. Female mosquitofish populations in the vicinity of pulp mills developed male gonopodia and showed malelike courtship activity and reproductive behavior (Bortone et al. 1989). In areas near Canadian pulp mills, Munkittrick et al. (1998) observed malelike secondary sex characteristics in 50% of female white suckers. Because genistein has been found in pulp mill effluent at a concentration of approximately 10 μg/L, isoflavones in BKME may contribute to these effects in feral fish.

The isoflavone compound equol was present in hog manure at concentrations of approximately 6 mg/L. Application of manure to fields resulted in runoff of equol into adjacent streams (Burnison et al. 2000). The concentration of equol exiting the drainage tiles within treated fields ranged from 10 to 1,300 ng/L, concentrations within the range that induced testis-ova in medaka (i.e., 400–800 ng/L). The recent data on the presence of genistein in the effluents of STPs in Germany (Spengler et al. 2001) indicate that STPs could also be a source of exposure of wild fish populations to isoflavone compounds.

Overall, this laboratory study with Japanese medaka indicates that isoflavone compounds should be considered candidate estrogenic compounds with the potential to impact gonadal differentiation and the development of secondary sex characteristics in feral fish populations. However, more work is required to determine whether these developmental responses in fish can be linked to population-related responses such as impairment of reproduction and recruitment. Further work should

be conducted to determine the concentrations of equol and genistein in various complex environmental mixtures, such as the effluents from STPs and pulp mills and the runoff from intensive agricultural operations.

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