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THE ESTROGENIC ACTIVITY OF CERTAIN PHYTOESTROGENS IN THE SIBERIAN STURGEON *ACIPENSER BAERI*

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Summary—Various phytoestrogens such as formononetin, daidzein, genistein and equol were synthesized. Their purity was assessed by various analytical techniques including melting point determination, thin-layer chromatography (TLC), infra-red spectra (i.r. spectra), nuclear magnetic resonance (^{1}H- and ^{13}C-NMR) and gas chromatography coupled with mass spectrometry (GC–MS). The estrogenic activity of these compounds, as well as biochanin A and coumestrol, was biologically tested by the induction of vitellogenin secretion in yearling sturgeon and compared to the activity of estradiol-17β.

Pure daidzein, biochanin A, genistein, equol and coumestrol all had estrogenic activity as assessed by their induction of hepatic synthesis of vitellogenin when administrated intraperitoneally to yearling Siberian sturgeon. Coumestrol seemed to be the most potent compound, inducing the most vitellogenin secretion with the lowest dose administered. Formononetin was inactive when administered by the intraperitoneal route. All the phytoestrogens tested were considerably less potent than estradiol-17β.

INTRODUCTION

The isoflavones formononetin, daidzein, biochanin A and genistein as well as the coumestan coumestrol (Fig. 1), are known to be present in various plant species, and particularly in soybean extracts and in vegetable dietary compounds like alfalfa [1–3]. They have been considered as responsible for the depression of fertility observed in sheep grazing clover pasture by acting on progesterone levels [4] or on pituitary luteinising hormone (LH) release [5, 6]. Their estrogenic activity has been assessed in biological assays such as the increase in the uterus weight of sheep [7], mouse [8] or rat [9] and by biochemical tests like binding to estrogen receptors [10, 11]. Several recent studies have shown a possible effect of these compounds on the synthesis of sex hormone-binding globulins (SHBG) by the liver, as well as on sex steroid metabolism [12, 13].

No data are available on the effect of these compounds on fish, even though the commercial diets routinely used for fish culture are known to be prepared using soybean extracts (up to 20–30% by weight) or alfalfa extracts or oil [14]. In these lower vertebrates, liver vitellogenin synthesis is an estrogen-dependent physiological process [15] which can be easily used as a test system for a wide variety of natural and synthetic estrogenic substances [16]. Vitellogenin, which is one of the eggs main yolk precursors in many oviparous vertebrates, is known to be synthesized in the liver of females during the reproductive season [17]. This hepatocyte secretion can be artificially induced in both sexes at any stage in the life cycle [18]. During research on cultured sturgeon in the South West of France, we obtained unexpected data concerned with elevated sex steroid and vitellogenin concentrations in the plasma of these fish [19]. A dietary estrogenic contamination was assumed to be responsible. Several steroidal estrogens were identified in the diet [19] but we also observed that vitellogenin synthesis could be induced in immature fish by the vegetable component (soybean) of the artificial diet [20]. In that study, vitellogenin levels were observed to rise compared to those in a control group of fish fed on a casein diet [20]. This result suggested that the estrogenic activity of phytoestrogens, such as isoflavones, isoflavans and coumestans,

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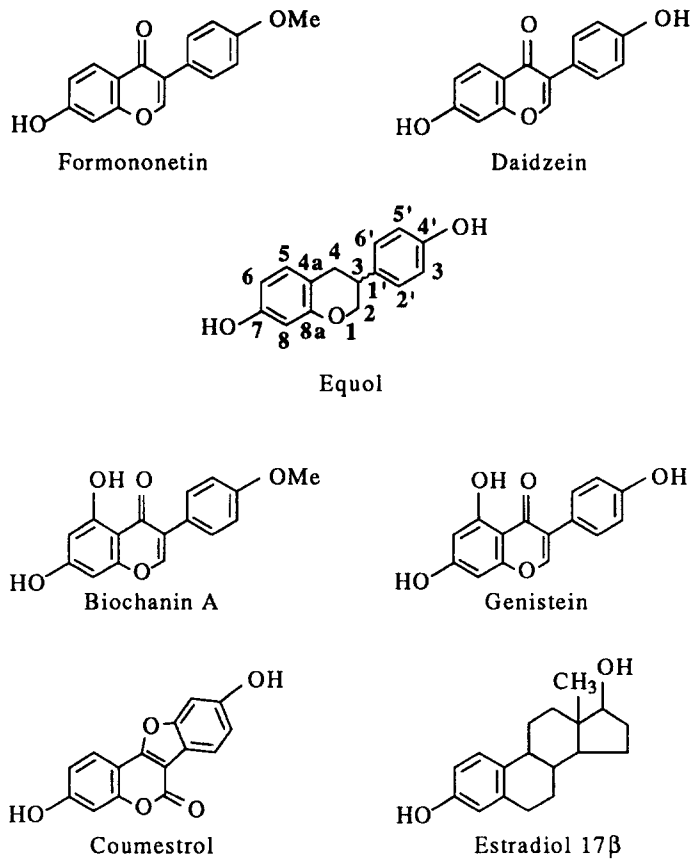

Fig. 1. Chemical structure of the phytoestrogens tested compared to the natural estrogen: estradiol-17β. The carbon atoms of equol are numbered to assist the interpretation of Table 1.

was responsible for the elevated vitellogenin levels.

The aim of this work is to establish whether these compounds could induce directly vitellogenin secretion in sturgeon.

EXPERIMENTAL

Synthesis of phytoestrogens

The isoflavones formononetin, daidzein and the isoflavan equol were synthesized according to Adlercreutz *et al.* [21] with a few modifications of the procedure for daidzein and equol synthesis. In all cases the development of the chemical reaction was assessed by TLC. The chemical reactions are presented in Fig. 2.

Formononetin was obtained by the method of Bass [22], by cyclization of 2,4-dihydroxy-4′ methoxybenzoin. This compound had been synthesized previously according to the methods of Chapman and Stephen [23] and Spoeri and Dubois [24] by condensation of resorcinol with 4-methoxy-phenyl acetonitrile.

Daidzein was obtained by demethylation of formononetin using BBr_3/CH_2Cl_2 as the demethylation agent. When we followed the procedure of Adlercreutz and coworkers [21], we obtained a mixture of formononetin, daidzein and various larger molecules of different R_f values in TLC. Daidzein was thus further purified by liquid chromatography on a silica gel column (1.6 × 70 cm). Under these conditions the recovery yield fell from 86 to 35%.

Equol was obtained by catalytic hydrogenation of daidzein on palladium/charcoal (Pd/C) as catalyst which reduced the ketone group and the double bond of the heterocycle. Equol was collected from the Pd/C-aquous ethanol mixture by 4 successive filtrations to remove the Pd/C residues. The ethanolic solution was then gently distilled under atmospheric pressure instead of vacuum evaporation reported by Adlercreutz and collaborators [21]. Equol was recrystallized from aqueous ethanol. This procedure allowed us to obtain the compound with a 35% yield.

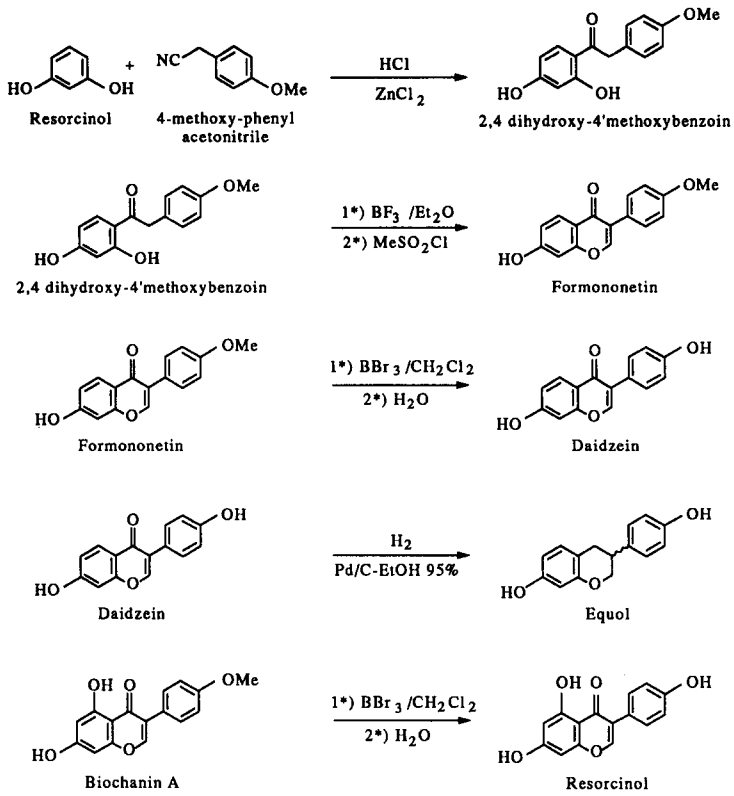

Fig. 2. Chemical synthesis reactions.

Biochanin A was purchased from Sigma (code No.: D2016) and was recrystallized in aquous ethanol before *in vivo* use or genistein synthesis.

Genistein was obtained by demethylation of biochanin A according to the same experimental procedure that was used for the transformation of formononetin into daidzein (yield 92%; the silica gel column chromatography was not necessary in this case).

Coumestrol was obtained from Apin Chemical Ltd (Abingdon, England). Estradiol-17β was purchased from Steraloid Ltd (Croydon, England).

Analytical methods

The products obtained by the synthesis procedures were analysed using various methods in order to confirm their chemical structure as well as assess their degree of purity. The cross-checking of all data obtained (melting point, TLC, i.r. spectra, ^{1}H- and ^{13}C-NMR spectra and GC–MS) allowed us to confirm the chemical structure of all phytoestrogens synthesized and to demonstrate a good degree of purity for each. Thus all synthesis procedures were continued on until a degree of purity of at least 99% was reached for each compound.

Melting point determination. Melting points were determined on a Kofler banc and compared to those previously published in the literature [25–27]. All the melting point information obtained was strictly comparable to those already reported in the literature, indicating a good degree of purity for each compound. These were: 258°C for formononetin, 323°C for daidzein, 153°C for equol, 213°C for biochanin A and 298°C for genistein.

Thin-layer chromatography (TLC). All thin-layer chromatography was performed on silica gel plates using chloroform–methanol (90:10; v/v) as eluant. Purification procedures were continued until we obtained a single spot for each recrystallized compound. R_f values were approximately: formononetin, 63; daidzein, 35; equol, 45; biochanin A, 66; genistein, 42 and coumestrol, 45, in accordance with those already cited by Braden *et al.* [7].

Infra red spectra (i.r.). The i.r. spectra were recorded as neat film nujol mulls on a Perkin–Elmer 1420 spectrophotometer. The i.r. profiles allowed us to check the functional groups of each molecule revealing aromatic rings, methyl-ether, hydroxyl, ketone groups, etc.

Nuclear magnetic resonance. ^{1}H and ^{13}C spectra were recorded on a Brucker AC 250

Table 1. Carbon-13 chemical shifts of equol in $(CD_3)_2SO$

	Carbon-13 Chemical shifts (ppm)											
	$C_{1'}$	$C_{2'-6'}$	$C_{3'-5'}$	C_2	C_3	C_4	C_{4a}	C_5	C_6	C_8	C_{8a}	$C_{4'}$ or C_7
Equol	131.67	128.3	115.3	70.27	37.17	31.31	112.47	130.10	108.10	102.50	154.53	156.14 or 156.50

The positions of the numbered carbon atoms are shown in Fig. 1.

spectrometer. The samples were dissolved in $(CD_3)_2SO$ or $CDCL_3$ according to solubility characteristics. The spectra obtained were interpreted and compared to those already published by other authors [27, 28] except for equol, since as far as we know its ^{13}C-NMR spectra has not been reported. The ^{13}C chemical shifts obtained for this compound are presented in Table 1. The interpretation of each spectra provided us with a better confirmation of the fine chemical structure of each compound than can be obtained by i.r. spectra.

Combined gas chromatography–mass spectrometry (GC–MS). Chromatographic analyses were performed on a Varian 3000 chromatograph equipped with a splitless injector and a 60 m × 0.22 mm ($\Phi = 0.1\ \mu$m) DB5 (J&B) capillary column. Each compound was derivatized in trimethylsilyl ether before analysis using an already prepared mixture of *N,O*-bis (trimethylsilyl) acetamid (BSA) purchased from Fluka (code No. 15242). The temperature program was as follows: $T = 50°C$, up to 160°C at 10°C per min then, from 160°C to 280°C in 2°C per min. Spectra were taken with a scan frequency of 2 scans/s. This chromatographic technique is more sensitive than TLC; it allows identification of trace contaminating compounds present at a 1% level. On each GC analysis, the phytoestrogens appeared as a single peak, allowing us to confirm the purity as well as the chemical stability of each compound (Fig. 3).

Mass spectra analyses were performed on a Finnigan TSQ 70 spectrometer using electron impact ionization (EI). For each compound the molecular ion was the most abundant ion,

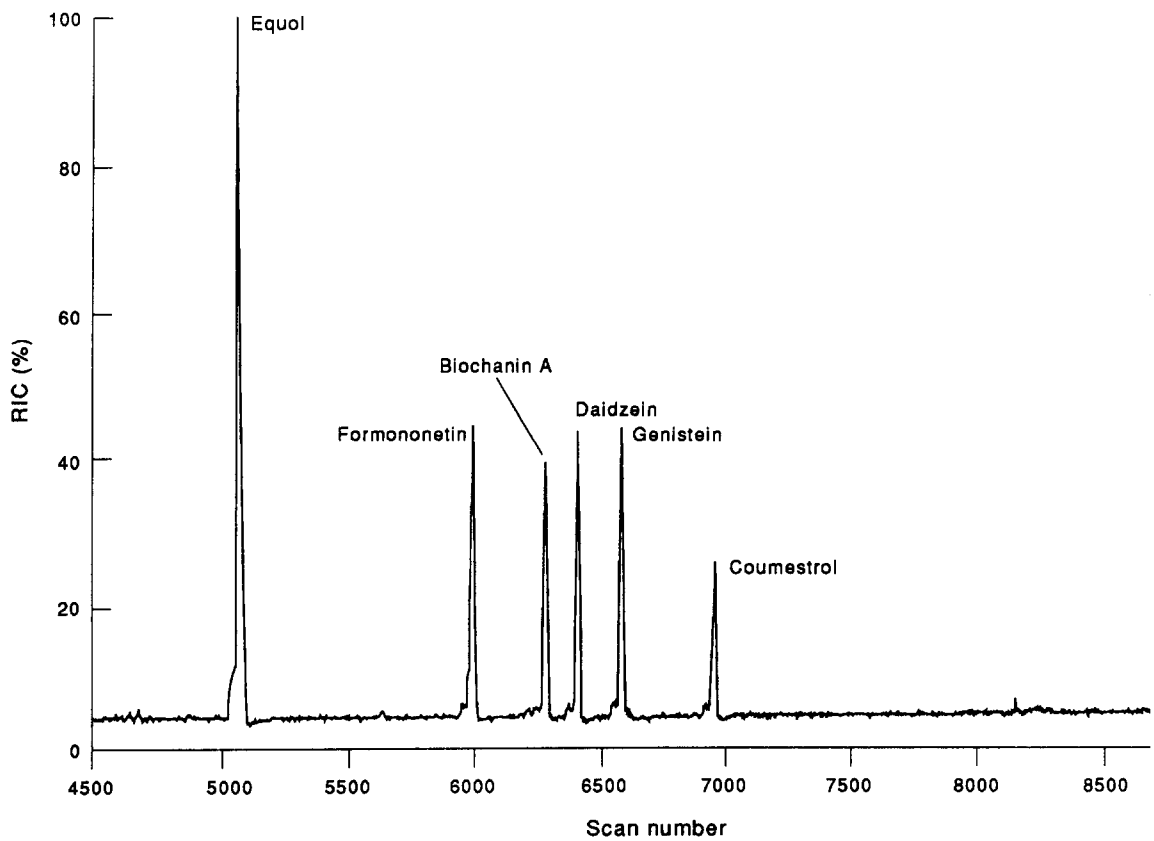

Fig. 3. Gas chromatography analysis of the six phytoestrogens used in the study. The phytoestrogens were mixed in CH_2Cl_2 and derivatized before the injection of 5 μl of the solution containing 1 mg/ml of each compound. For more details see 'Analytical Methods'.

except for equol for which the main ion was $m/z = 192$, which is in agreement with previous studies [29, 30].

Experimental methods

Animals. The experiment was carried out on yearling Siberian sturgeon of 20 g mean weight and 12 cm mean length. They were hatched in the experimental fish farm of Donzacq (Landes). Three weeks before the beginning of the experiment, fish were fed with natural prey (diptera larvae and silkworms) which avoided prior estrogenic contamination through commercial fish food, as has been demonstrated previously. They were separated into eight batches of four fish each and held in identical running water conditions. During the whole experiment, fish were fed with natural prey.

Experimental procedure. Before beginning the experiment, ten randomly chosen fish were blood sampled in order to determine the initial vitellogenin level. For intraperitoneal administration all phytoestrogens, and estradiol as well, were suspended in a solution of 3% benzylic alcohol in gelatinized phosphate-buffered saline (0.05; pH 7.4; gelatin 1%; NaCl 0.9%). Each fish received 0.5 ml of solution per injection, including the control fish which were treated with the injection solution alone. Injected doses varied from one phytoestrogen to another, based on previous data obtained by Braden *et al.* [7] in ewes. Formononetin and biochanin A were used at 0.5 mg/g of body weight, daidzein and genistein at 0.2 mg/g of body weight, equol and coumestrol at 0.05 mg/g of body weight and estradiol-17β at 0.0001 mg/g of body weight. Four successive injections were performed on alternate days over 8 days. On the tenth day, blood was collected through the caudal vein using heparinized syringes, centrifuged at 10,000 *g* for 5 min and the plasma stored at $-20°C$ until assayed for vitellogenin.

Plasma analysis. Vitellogenin was measured in plasma by homologous enzyme linkage immuno-sorbent assay (ELISA) [20, 31]. Specific vitellogenin immunoserum was raised in a rabbit against purified Siberian sturgeon vitellogenin prepared by two successive chromatographic steps [32]. All immunological steps were performed in phosphate-buffered saline (0.01 M; pH 7.4; 0.9% NaCl) containing 2% swine serum. Secondary antibodies and PAP complexes were purchased from Dako (Denmark). Peroxidase of the PAP com plexes was revealed by O-phenylene diamine in citrate-phosphate buffer (0.1–0.2 M, pH 5) and staining was stopped by 4 M H_2SO_4. The staining reaction was measured at 495 nm on a titertec MKII Multiscan Plus spectrophotometer. Inter-assay variation was 11% ($n = 30$), intra-assay variation was 10% ($n = 40$). According to our method, the lower limit of sensitivity of the assay was 0.1 μg of vitellogenin per ml of plasma.

Table 2. Vitellogenin levels induced by intraperitoneal injection of phytoestrogens in yearling Siberian sturgeon (20 g mean weight)

Estrogenic compounds	Dose (mg/g)	Vitellogenin (μg/ml)	No. of fish
Control	—	<0.1	($n = 5$)
Formononetin	0.5	<0.1	($n = 4$)
Daidzein	0.2	2 ± 0.06	($n = 4$)
Equol	0.05	8.8 ± 2.8	($n = 3$)
Biochanin A	0.5	98 ± 14	($n = 4$)
Genistein	0.2	213 ± 56	($n = 4$)
Coumestrol	0.05	272 ± 98	($n = 4$)
Estradiol-17β	0.0001	246 ± 97	($n = 4$)

RESULTS

Vitellogenin was undetectable in the plasma of ten randomly chosen fish at the beginning of the experiment. Plasma concentrations of vitellogenin obtained after treatment are presented in Table 2. Compared to the control fish all the phytoestrogens tested, except formononetin, induced vitellogenin synthesis. Biochanin A, administrated at a dose of 0.5 mg/g of body weight, induced a plasma vitellogenin level of 98 ± 14 μg/ml. Daidzein induced a plasma vitellogenin level of 2 ± 0.06 μg/ml, and genistein a level of 213 ± 56 μg/ml, when administrated at a dose of 0.2 mg/g of body weight. Equol induced a plasma vitellogenin level of 8.8 ± 2.8 μg/ml and coumestrol of 272 ± 98 μg/ml, when administrated at a dose of 0.05 mg/g of body weight. Estradiol injected at a dose of 0.0001 mg/g of body weight induced a plasma vitellogenin level of 246 ± 97 μg/ml.

DISCUSSION

Because of the large amount of phytoestrogens needed for this type of *in vivo* experiment, the bioassay was performed on a restricted number of fish per batch using only one dose, which was based on tests by other authors conducted on ewes [7].

The secretion of vitellogenin in response to estradiol is dose-dependent [33] and thus it can be used to assess the estrogenicity of potential estrogenic compounds like phytoestrogens.

However, the results obtained in this system must be considered cautiously since several previous studies have demonstrated that the estrogenic potency of such substances can vary from one species to another [11] and also from one administration route to another [7]. Nevertheless, all the phytoestrogens tested, except formononetin, induced vitellogenin secretion; this indicates that phytoestrogens are able to bind to the estrogen receptors involved in vitellogenin secretion. According to the literature [7, 34] the estrogenicity of isoflavones is highly dependent upon the stereochemical structure, particularly the distance between the two hydroxylated groups carried by the two aromatic nuclei and the angle of "twist" of the two hydroxylated rings with respect to each other. Some compounds have a stereochemical structure closer to the natural hormone estradiol-17β than others and are therefore likely to be the most potent.

The inactivity of formononetin was expected. Indeed, various authors have reported that its estrogenic activity in mammals is related to its prior metabolism by intestinal bacteria flora to daidzein and equol [35, 36]. Braden *et al.* [7] have already shown the lack of activity of this compound when employed by intramuscular or intraperitoneal route. The only positive results were obtained on ewes by intra-ruminal administration. In the same study coumestrol was demonstrated to be 10 times more active than genistein, which itself was 2 times more active than biochanin A. Wong and Flux [8] also assessed the estrogenic potencies of genistein, biochanin A, daidzein and formononetin, using the mouse uterine assay, after oral administration of each phytoestrogen. They found that the relative activities of these isoflavones were: genistein 1.5, biochanin A 1.0, daidzein 0.4 and formononetin "slight activity". Shutt and Cox [37] discussed the relative molar binding affinities (RMB) of isoflavones to sheep uterine estrogen receptors; they were: coumestrol 5, genistein 0.9, equol 0.4, daidzein 0.1, biochanin A, 0.01, formononetin, 0.01. Then in various other studies authors have considered that coumestrol is 1000-fold less active as estradiol-17β itself [5] and that equol binds 1000 times [10] or 100 times [11] less avidly than estradiol-17β to uterine estrogen receptors in rat. In these two systems equol was considered to act essentially as an estrogen antagonist because of the lability of its link to the estrogen receptors [10, 11].

Even though a relative scale of estrogenic potency cannot be constructed in our case because of the employment of only a single dose which was different depending on the phytoestrogen, our results seem to be in accordance with these previous data obtained in mammals. In our case however, phytoestrogens must be considered as estrogen agonist since they induced a vitellogenic response. These results suggest that our previous finding of high vitellogenin levels in the plasma of immature Siberian sturgeon fed a soybean diet [20] may have been caused, at least in part, by the estrogenic activity of phytoestrogens present in the diet.

Now that the estrogenic activity of these compounds has been demonstrated by the induction of vitellogenin secretion, it will be interesting to test them on other estrogen-dependent functions like steroid binding protein (SBP) synthesis and gonadotrophic hormone (GTH) release by pituitary cells. Indeed, previous work has demonstrated that the action of these compounds can vary from one species to another and also from one target tissue to another [10, 11].

This estrogenic effect, directly linked to the diet, could be of considerable consequence in fish farming due to the important role of estrogens in physiology. In fish, the daily intake of estrogenic compounds, via the diet, could act on various physiological processes such as reproduction, growth and osmoregulation [19]. This dietary estrogenic contamination must be kept in mind, especially when it is realized that in the near future it is hoped that vegetable protein souces like soybean or alfalfa will replace animal protein in fish diets.

In summary, the estrogenic potency of phytoestrogens in fish has been directly demonstrated for the first time, supporting previous results obtained in sturgeon fed a semisynthetic diet containing soybean [20]. However, the variability in action of these compounds from one target tissue to another, as well as from one species to another, implies that other studies must be performed before generalizing to other fish species. This dietary contamination suggests that there may be environmental problems if estrogenic substances are present in fish farm effluent which then enters a natural water-source.

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REFERENCES

1. Walter E. D.: Genistin (an isoflavone glucoside) and its aglucone genistein, from soyabean. *J. Chem. Soc.* **23** (1941) 3273–3276.
2. Bradbury R. B. and White D. E.: Estrogens and related substances in plants. *Vitam. Horm.* **12** (1954) 207–233.
3. Axelson M., Sjovall J., Gustafsson B. E. and Setchell K. D. R.: Soya a dietary source of the non-steroidal oestrogen equol in man and animals. *J. Endocr.* **102** (1984) 49–56.
4. Obst J. M. and Seamark R. F.: Plasma progesterone concentrations during the reproductive cycle of ewes grazing yarloop clover. *J. Reprod. Fert.* **21** (1970) 545–547.
5. Leavitt W. W. and Wright P. A.: The plant estrogen coumestrol as an agent affecting hypophysial gonadotropic function. *J. Exp. Zool.* **160** (1965) 319–327.
6. Findlay J. K., Buckmaster J. M., Chambley W. A., Cumming I. A., Hearnshaw H. and Goldin J. R.: Release of luteinising hormone by estradiol-17β and a gonadotrophin releasing hormone in ewes affected with clover disease. *Neuroendocrinology* **11** (1973) 57–66.
7. Braden A. W. H., Hart N. K. and Lamberton J. A.: The oestrogenic activity and metabolism of certain isoflavones in sheep. *Aust. J. Agric. Res.* **18** (1967) 335–348.
8. Wong E. and Flux D. S.: Oestrogenic activity of red clover iso-flavones and some of their degradation products. *J. Endocr.* **24** (1962) 341–348.
9. Perel E. and Lindner H. R.: Dissociation of uterotrophic action from implantation activity in two non-steroidal oestrogens (coumestrol and genistein). *J. Reprod. Fert.* **21** (1970) 171–175.
10. Tang B. Y. and Adams N. R.: Effect of equol on oestrogen receptors and on synthesis of DNA and protein in the immature rat uterus. *J. Endocr.* **85** (1980) 291–297.
11. Thompson M. A., Lasley B. L., Rideout B. A. and Kasman L. H.: Characterisation of the oestrogenic properties of a non-steroidal oestrogen equol, extracted from the urine of pregnant macaques. *Biol. Reprod.* **31** (1984) 705–713.
12. Adlercreutz H., Höcherstedt K., Bannwart C., Bloigu S., Hämäläinen E., Fotsis T. and Ollus A.: Effect of dietary components, including lignans and phytoestrogens, on enterohepatic circulation and liver metabolism of estrogens and on sex hormone binding globulin (SHBG). *J. Steroid Biochem.* **27** (1987) 1135–1144.
13. Goldin B. R., Adlercreutz H., Gorbach S. L., Woods M. N., Dwyer J. T., Conlon T., Bohn H. and Gershoff S. N.: The relationship between estrogen levels and diets of the Caucasian American and Oriental immigrant women. *Am. J. Clin. Nutr.* **44** (1986) 945–953.
14. Barrucand M., Ferlin P., Lamarque P. and Sabaut J. J.: Alimentation artificielle de l'esturgeon sibérien *Acipenser baeri. Proc. World Symp. Fin. Nutr. Fish. Tech.* **1** (1979) 411–421.
15. Ng T. B. and Idler D. R.: Yolk formation and differenciation in teleost fishes. In *Fish Physiology IX* (Edited by W. S. Hoar, D. J. Randall and E. M. Donaldson). (1983) pp. 373–404. Academic Press, New York.
16. Vaillant C., Le Gellec C., Pakdel F. and Valotaire Y.: Vitellogenin gene expression in primary culture of male rainbow trout hepatocytes. *Gen. Comp. Endocr.* **70** (1988) 284–290.
17. Wallace R. A., Jared D. W. and Nelson B. L.: Protein incorporation by isolated amphibian oocytes. *J. Exp. Zool.* **175** (1970) 259–270.
18. Wallace R. A. and Selman K.: Cellular and dynamic aspects of oocyte growth in teleosts. *Am. Zool.* **21** (1981) 325–343.
19. Pelissero C., Cuisset B. and Le Menn F.: The influence of sex steroid in commercial fish meals and fish diet on plasma concentration of estrogens and vitellogenin in cultured Siberian sturgeon *Acipenser baeri. Aqua. Liv. Ressour.* **2** (1989) 161–168.
20. Pelissero C., Le Menn F. and Kaushik S.: Estrogenic effect of dietary soyabean meal on vitellogenesis in cultured Siberian sturgeon *Acipenser baeri. Gen. Comp. Endocr.* (1991) (In Press).
21. Adlercreutz H., Musey P. I., Fotsis T., Bannwart C., Wähälä K., Mäkelä T., Brunow G. and Hase T.: Identification of lignans and phytoestrogens in urine of chimpanzees. *Clin Chim. Acta* **158** (1986) 147–154.
22. Bass R.: Synthesis of chromones by cyclisation of 2-hydroxyphenyl ketones with boron trifluoride-di-ethyl ether and methanesulphonyl chloride. *J. Chem. Soc. Chem. Commun.* **2** (1976) 78–79.
23. Chapman E. and Stephen H. L.: Di- and Tri-hydroxydeoxybenzoins. *J. Chem. Soc.* **123** (1923) 404–409.
24. Spoeri P. E. and Dubois A. S.: The Hoesh synthesis. *Org. React.* **5** (1949) 387–412.
25. Walz E.: Isoflavan und saponin-glucoside is soja hispida. *Justus Liebig's Annalen Chemie* **489** (1931) 118–155.
26. Baker W., Chadderton J., Harborne J. and Ollis W. D.: A new synthesis of isoflavone. Part I. *J. Chem. Soc.* (1953) 1852–1860.
27. Pelter A., Ward R. S. and Bass R. J.: The carbon—13 nuclear magnetic resonance spectra of isoflavones. *J. C. S. Perkin I* (1978) 666–668.
28. Jha H. C. and Zilliken F.: Carbon—13 chemical shift assignments of chromones and isoflavones. *Can. J. Chem.* **58** (1980) 1211–1219.
29. Axelson M., Kirk D. N., Farrant R. D., Cooley G., Lawson A. M. and Setchell K. D. R.: The identification of the weak estrogen equol [7hydroxy-3(4'hydroxyphenyl)chroman] in human urine. *Biochem. J.* **201** (1982) 353–357.
30. Bannwart C., Adlercreutz H., Wähälä K., Kotiaho T., Hesso A., Brunow G. and Hase T.: Identification of the phytoestrogen 3',7-dihydroxyisoflavan, an isomer of equol in human urine and cow's milk. *Biomed. Env. Mass. Spectrom.* **17** (1988) 1–6.
31. Cuisset B., Pelissero C., Le Menn F. and Nunez Rodriguez J.: ELISA for *Acipenser baeri* vitellogenin. *Proc. First. Int. Symp. Acipenser* (1989).
32. Pelissero, C.: Interraction de l'alimentation sur la presence de stéroides, d'isoflavones et de leurs dérivés dans le plasma de l'esturgeon sibérien *Acipenser baeri.* Conséquences sur la vitellogénèse. *Th. Univ. Bdx I* **427** (1990) p. 98.
33. Van Bohemen C. G., Lambert J. G. D. and Van Oordt P. T.: Vitellogenin induction by estradiol in estrone primed Rainbow trout *Salmo gairdneri. Gen Comp. Endocr.* **46** (1982) 136–139.
34. Bickoff E. M., Livingston A. L. and Booth A. N.: Estrogenic activity of coumestrol and related compounds. *Arch. Biochem. Biophys.* **88** (1960) 262–266.
35. Batterham T. J., Hart N. K., Lamberton J. A. and Braden A. W. H. Metabolism of oestrogenic isoflavones in sheep. *Nature* **206** (1965) 609.
36. Batterham T. J., Shutt D. A., Hart N. K., Braden A. W. H. and Tweedale H. J.: Metabolism of intraruminally administrated [4-^{14}C] formononetin and [4-^{14}C] biochanin A in sheep. *Aust. J. Agric. Res.* **22** (1971) 131–138.
37. Shutt D. A. and Cox R. I. Steroid and phytoestrogen binding to sheep uterine receptors *in vitro. J. Endocr.* **52** (1972) 299–310.