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Effect of Genistein-Enriched Diets on the Endocrine Process of Gametogenesis and on Reproduction Efficiency of the Rainbow Trout Oncorhynchus mykiss

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Abstract: Three practical diets were formulated to contain 0, 500, or 1000 ppm genistein. The three diets were distributed for 1 year to groups of rainbow trout undergoing their first gametogenesis and until spawning. Growth performance of rainbow trout was not affected by dietary treatments. Plasma cholesterol levels were equivalent between groups. In males, a slight but constant induction of vitellogenin (VTG) synthesis and a decrease in testosterone levels were observed. A slight decrease in plasma levels of βFSH and βLH was noticed at the end of spermatogenesis in the male fish fed a diet with 500 ppm (genistein) (from 2.16 ± 0.39 to 1.47 ± 0.23 for βFSH and from 0.44 ± 0.09 to 0.31 ± 0.09 for βLH). There was a significantly reduced 17α,20β(OH)2-progesterone (from 10.93 ± 0.88 in control to 5.46 ± 0.92 in males and from 251.22 ± 21.40 to 183.22 ± 13.48 in females). Testicular development was accelerated in genistein-fed fish, and sperm motility and concentration were decreased in a dose-dependent manner at spawning. In females, a significant increase in plasma VTG occurred only at the beginning and at the end of oogenesis. Testosterone levels were decreased at the beginning of oogenesis. Both βFSH and βLH were decreased by genistein (from 6.38 ± 1.55 to 3.44 ± 0.82 for βFSH and from 15.18 ± 3.00 to 6.93 ± 0.99 for βLH in females), whereas spawning was delayed only in females fed the diet with 500 ppm of genistein. Gamete quality was impaired only in this group, as underlined by a lower percentage of ovulating females (from 100 to 79% at the end of the trial), a lower fertilization rate, and a lower viability of fry. These results may be explained by the agonistic/antagonistic effect of genistein on estrogen function related to the tissue ratio between endogenous estrogens/genistein.

Keywords: genistein; trout; steroid; vitellogenin; GTH; reproduction; diet

Genistein (genistein) is a phytoestrogen whose properties are numerous. It binds to the estradiol receptor (ER) in mammals (Martin *et al.*, 1978) and fish (Latonnelle *et al.*, 1999). It is an anti-oxidant (György *et al.*, 1964) and a protein kinase C (PKC) and a protein tyrosine kinase (PTK) inhibitor (Osada *et al.*, 1988 ; Akiyama *et al.*, 1987). Soybean, widely used in animal and human nutrition is rich in phytoestrogens and especially in genistein. Numerous other vegetables are also known to contain such compounds (Farnsworth *et al.*, 1975 ; Setchell, 1985). The estrogen agonist/antagonist effect of genistein is well known (Pike *et al.*, 1999). This compound is known to bind to estradiol receptors in the uterus and to be uterotrophic (Hopper *et al.*, 1998 ; Milligan *et al.*, 1998). It prevents the full pituitary gonadotropin (GTH) release (Hughes, 1988) and acts on sexual differentiation in mammals (Levy *et al.*, 1995). Impairments of progesterone synthesis *in vitro* (Kaplanski *et al.*, 1981) and *in vivo* (Awoniyi *et al*., 1998) by high doses of genistein are also reported. However very few data are available on the *in vivo* effects of administration of this compound during the entire reproductive cycle. When they exist they report studies on mammals and more precisely in rats (Awoniyi *et al.*, 1998 ; Casanova *et al.*, 1999) on *in utero* and neonatal exposures.

Trout (*Oncorhynchus mykiss*), a teleost fish, is a convenient model to assess this effect because of rather long gametogenesis normally lasting a year. Moreover its reproductive function has been thoroughly studied (Hoar *et al.*, 1983) at a time when soy was not a major component of fish food. It offers the advantage of a late sexual differentiation at the fry stage whereas this developmental step occurs *in utero* in mammals. Even more, this development is much easier to monitor in reproducible conditions in a large number of individuals. Compared to mammals (Torbjorn, 1995), the degradation of xenobiotics is likely to be less important since the influence of the gut microflora is nil in this fish (Lesel, 1987). Indeed, it is known that daidzein can be transformed into equol by the gut microflora of many mammals including

78 humans (Setchell and Cassidy, 1999) and that equol is about 10 times more estrogenic than
79 daidzein (Pelissero *et al.*, 1990). This particular situation is of interest when the estrogenic
80 effect is approached. In addition, many tools are now available to assess the action of
81 endocrine disruptors in this species. The estrogenicity of genistein in trout has already been
82 demonstrated *in vitro* in hepatocyte cultures at both the transcriptional and post-
83 transcriptional levels (Pelissero *et al.*, 1993 ; Bennetau - Pelissero *et al.*, 1998). Its binding to
84 Steroid Binding Protein (SBP) has been studied in man (Martin *et al.*, 1995) and in fish
85 (Bennetau - Pelissero *et al.*, 1998). *In vitro*, genistein used at high concentrations, acts as a
86 low aromatase inhibitor in the ovary of rainbow trout (Pelissero *et al.*, 1996).

87 The aim of the present study is to determine *in vivo* and, in rainbow trout over a full
88 reproductive cycle, the effect of dietary genistein on the endocrine physiology and
89 reproductive function. This long-term study was initiated to analyse the complex sequential
90 effects of an endocrine disruptor upon the reproductive function and to provide baseline data
91 for future work on the effects of genistein at the different levels of the estrogenic information
92 steps.

93

93 # MATERIAL AND METHODS

94 ***Fish.***

95 A mixed population of rainbow trout originating from our own stock (INRA) were used. They
96 were 6-month-old and had a mean-weight of 40g at the beginning of the experiment. They
97 were allotted into 6 groups of 100 fish in 6 different tanks. Fish were grown at a constant
98 water temperature of 17°C ± 1°C at the experimental fish farm (INRA, Donzacq, France)
99 during the first part of the experiment. Three months before spawning they were transferred to
100 another experimental fish farm (INRA, Lees Athas, France), and kept at a constant water
101 temperature of 7°C. They were maintained under natural light cycle during the whole
102 experiment.

103

104 ***Diets.***

105 Genistein (250 g) was obtained with an overall yield of 70%, by a similar process described
106 for daidzein (Pelissero *et al.*, 1990) using phloroglucinol as starting material. The
107 spectroscopic (NMR ^{1}H and ^{13}C, IR) and physical data (CCM, melting point) fit those of the
108 literature (Waltz, 1931 ; Baker *et al.*, 1953 *;* Pelter *et al.*, 1978). Three practical diets with fish
109 meal as the major protein source were prepared with three concentrations (0, 500 and 1000
110 ppm) of genistein (see table 1). Diets were distributed *ad libitum* daily and twice a day to
111 visual satiety. Fish were kept unfed for 36 hours before every sampling.

112

113 ***Sample timing.***

114 The first sampling (T0) was 3 days before the beginning of the feeding trial. Then samples
115 were withdrawn at about ten weeks intervals (figure 1).

116

117 ***Blood samples.***

Fish were weighed at each sampling time, and each time 20 fish per tanks were killed by a sharp blow on the head for gonad examination. Blood samples were withdrawn from anaesthetised fish (ethylen glycol monophenyl ether 0.3%) from the caudal vessels using an heparinized syringe. Blood was immediately transferred into heparinized vial stored on ice. And then centrifuged at 6000 g for 10 minutes plasma were stored at -20°C until hormone measurements. At each sampling time, whole body, liver, gonad, and eviscerated body weight were recorded.

Cholesterol analysis

Plasma cholesterol analysis was performed on samples collected at T3 in July. Blood was withdrawn from the caudal vessels into heparinized tubes. Plasma was collected after centrifugation at 6000 g for 10 min and stored at -20°C until analysed. Total plasma cholesterol was determined by colorimetric enzymatic method using a commercial kit (method CHOD-PAP, Boehringer Mannheim GmbH, Germany).

Vitellogenin

Plasma vitellogenin (VTG) levels were measured by a specific ELISA technique (Bon *et al.*, 1997).

Plasma concentration of steroid analysis.

Testosterone (T), estradiol (E_2) and 11-ketotestosterone (11KT) were monitored during the whole experiment. 17α,20β$(OH)_2$-progesterone (17α,20β$(OH)_2$-P) was measured only at T5. Steroid analyses were performed on ethyl-acetate - cyclohexane (v-v) extracts from plasma samples (Fostier *et al.*, 1979). Plasma 11-KT levels were measured according to Cuisset *et al.*, (1995). E_2, T and 17α,20β$(OH)_2$-P levels were measured using ELISAs raised on specific

143 antibodies kindly donated by Dr Kime (University of Sheffield, UK), Dr Zanuy (CSIC
144 Castellon, Spain) and Dr Fostier (INRA Rennes, France).
145 The technique is extensively described in (Cuisset *et al.*, 1995).
146

147 ***Gonadotrophins (GTHs)***

148 Plasma GTH1 (FSH) and GTH2 (LH) analysis were done by B. Breton (INRA, Rennes,
149 France) according to the method described by Govorum *et al.* (1998).
150

151 ***Histological analysis.***

152 Gonadal samples were examined histologically (Martojas and Martojas, 1967). The
153 histological staining followed the Masson's trichrome technique. In females, 4 ovarian stages
154 were identified whereas in males 5 stages were identified (see table 2). Gonad samples were
155 analysed only from sampling times T0 to T3.
156

157 ***Spawning.***

158 Individually identified (Spaghetti tag, COFA) fish were reared for 2 months before spawning
159 in the experimental fish farm (INRA, Lees Athas, France) at a temperature of 7°C. One and a
160 half months before spawning, males were checked for spermiation. One month before
161 spawning, females were checked weekly for ovulation. First ovulations started on the 22nd
162 December and proceeded for a month and a half. Eggs and milt from the same dietary groups
163 were used to produce progeny. Fish were weighed at spawning. Sperm was collected by
164 stripping. Milt volume collected by stripping was measured in test tubes and motility assessed
165 in water under a x40 magnification. Milt concentration was measured by spermatocrit
166 measurement on a hematocrit rotor of a centrifuge Sigma 1-13. Females were stripped every
167 week and selected on their ability to release their eggs. The date of spawning was recorded.

Egg diameter, weight of ovulated egg, weight of 100 eggs and weight of residual gonad were recorded for each female at spawning. Fertilisation was performed mixing 500 eggs from a given female with 1 ml of a mixture of milt from at least three males. It was diluted 1:30 with the INRA dilution mixture (Labbé and Maisse, 1996). After fertilisation, viability of the eggs, survival at day 1 and survival at embryo stage were recorded.

Statistical analysis.

After ANOVA, mean comparisons were made between the duplicate tanks and between the treatments. These tests were performed at each sampling time on the whole population and on the females and males separately. Mostly a T test was used and the acceptability threshold was fixed to $\alpha = 0.05$. Sometimes, because of unequal sex ratio in the randomly harvested samples, a non parametric Kruskal-Wallis test was performed with the same significance threshold. These tests allowed the pooling the of fish from duplicate tanks since no significant difference was found at any time for any parameter. The frequency distributions were compared using the exact method of Fisher.

RESULTS

Growth

No incidence of dietary genistein levels was noted on fish weight during the whole experiment and no difference was noted between males and females. At the beginning of the experiment mean weight was 41.8 ± 1.4 g and 40.8 ± 2.0 g for male and female respectively. At the end of the trial mean male weights were 800.1 ± 44.9, 891.9 ± 39.8 and 852.8 ± 29.3 for 0, 500ppm and 1000 ppm genistein exposed fish respectively. Mean female weights were 753.9 ± 28.2, 802.1 ± 31.1 and 792.6 ± 23.1 for 0, 500ppm and 1000 ppm genistein fed fish respectively.

193

194 ***Cholesterol levels in plasma***

195 The analysis was performed on vitellogenic females and males undergoing spermiogenesis.
196 No differences were recorded between treatments with the mean values (ng/ml) ± SEM as
197 follows : 0 ppm : 5.15 ± 0.20 (n=25); 500 ppm : 5.71 ± 0.35 (n=26); 1000 ppm : 5.72 ± 0.38
198 (n=31).

199

200 ***Steroids and VTG in males***

201 Control fish exhibited low levels of plasma estradiol (E_2) i.e. below 1 ng per ml during the
202 whole experiment except at T4 when the levels rose to 3.2 ng/ml. The genistein-fed fish did
203 not exhibit the same E_2 increase (significant difference $\alpha = 0.05$). At spawning time, (T5),
204 there was still a significant difference although reduced (figure 2a).

205 As shown in figure 2b, dietary genistein significantly increased plasma VTG levels in
206 males at all sampling times except T5. However, despite a 2 to 3 fold difference, the plasma
207 VTG levels in the genistein-fed fish remained low (<3µg/ml) compared to VTG levels in the
208 females (figure 3b).

209 After a period during which the plasma testosterone (T) levels were below 5 ng/ml a
210 marked increase (50 ng/ml) was noted at T4 corresponding to the stage of full spermiogenesis
211 in all groups. At T5, the plasma T levels decreased (figure 2c). Plasma T levels were always
212 significantly lower in genistein-fed fish than in control except at T3 and T4 during
213 spermatogenesis.

214 11KT was the major androgen present during spermiogenesis. The plasma 11KT
215 levels were generally not different between treatments ; they were significantly lower in
216 genistein-fed fish than in the control at T1 and T5 (spawning) where there was a significant
217 dose-dependant decrease with increasing levels of genistein in the diet (figure 2d).

218

219 ***Steroids and VTG in females***

220 Until T2, plasma E_2 levels were initially below 1 ng/ml. A slight increase occurred
221 between T2 and T3 reaching 4 ng/ml. Then, between T3 and T4, there was a marked increase
222 up to 35 ng/ml during full vitellogenesis. At spawning, the plasma E_2 levels decreased in all
223 fish (figure 3a). There were no significant differences between control and treated fish at any
224 time.

225 Plasma VTG levels in female trout started to increase from early vitellogenesis
226 onwards (<100 µg/ml) to reach levels of 180 mg/ml by spawning. They were significantly
227 higher in genistein-fed-fish at T2 (early vitellogenesis) and at T5 (spawning) (figure 3b).

228 Plasma T levels in fish fed with a diet containing 1000 ppm of genistein diet were
229 significantly lower than in control at the beginning of the experiment (T1 : KB1 = 0.45 ±
230 0.11; KB2 : = 0.28 ± 0.09; KB3 = 0.16 ± 0.03 and at T2 : KB1 = 0.79 ± 0.23; KB2 = 0.61 ±
231 0.18; KB3 = 0.39 ± 0.09). However, at T4, during full vitellogenesis, because of great inter-
232 individual differences, the effect was no longer significant. At spawning, the pattern was
233 reversed since T was higher when dietary genistein increased. Significant differences were
234 observed only between control and 1000 ppm genistein treated fish (figure 3c).

235 Plasma 11KT levels were low and did not vary between treatments except at T4.
236 Generally in this species, this steroid is absent from the female plasma, but even in control
237 fish, the 11KT plasma levels reached concentrations above 2 ng/ml at T4 and T5. In treated
238 fish this rise was delayed and appeared only at T5 (figure 3d).

239

240 ***Gonadotrophin and 17α,20β(OH)$_2$-P levels***

241 In males, plasma βLH levels were very low at T4 (below the assay detection limit of
242 0.1ng/ml) and at T5 (below 0.6 ng/ml). For both βFSH and βLH, no significant differences

were detected between control and treated fish either at T4 or at T5. However the mean value of both hormones were consistently lower for the 500 ppm genistein fed fish at these two sampling times (table 3).

In females, plasma βFSH levels declined consistently between T4 and T5 only in genistein-fed fish. At T4, plasma βFSH levels were significantly lower in the 1000 ppm treated fish than in the control. At that time the plasma βLH levels were very low (below 0.4 ng/ml). At spawning (T5), the βLH levels were much higher than at T4 but were consistently lower ($\alpha = 0.05\%$) in genistein-fed fish (table 3).

In males, the pattern of changes in $17\alpha,20\beta(0H)_2$-P was comparable to those of βFSH and βLH (table 3). However, plasma $17\alpha,20\beta(0H)_2$-P levels in the 500 ppm treated group were significantly lower than in control fish. In females, the changes in $17\alpha20\beta(OH)_2$-P was comparable to plasma βLH levels. Although the difference was significant in 500 ppm treated females ($p<0.05$), it was not in 1000 ppm treated females.

Gonadal development

In males, at T0 the only stage that could be identified in the testis was spermatogonia. At T1, both spermatogonia of type 1 and 2 were distinguishable through histological observations. This corresponded to stage 1 (table 2). At T2, the analysis exhibited differences between the three groups. Briefly, it appeared that the more genistein in the diet the higher percentage of males at stage 4 and the lower percentage of males at stage 1, 2 and 3 (figure 4a). At T3 similar results were obtained although less distinctly (figure 4b). The highest percentage of males at T4 was recorded in the 1000 ppm treated group whereas in this group there were no stage 3 males, although there were among control and 500 ppm treated fish. However, as per the Fisher test of frequency comparison, the differences recorded were not significant.

In females no effect of any treatment (presence or absence of genistein) could be identified. At T0 only very small oogonia cells were seen which were not distinguishable from the male cells. At T1, the larger cells observed were at stage 2 in all fish. At T2, females were essentially at stage 2. At T3 all females had reached stage 3 since in all females small VTG granules were identified at the periphery of the bigger oocytes whereas the zona-radiata was already large and presented at least two distinct layers. No samples were withdrawn for microscopic observations at T4 and T5 (spawning period).

Gamete quality

Data on various parameters related to male gamete quality are presented in Table 5. The percentage of male in spermiation on 29th October (between T4 and T5) increased with genistein intake, although the difference was not significant. This concurs with the results obtained by histological analysis. The GSI measured after sperm collection was not modified either in control or in treated fish. The sperm volume collected at stripping increased significantly with genistein consumption. Spermatocrit was significantly decreased in trout fed diets with 1000 ppm of genistein. Sperm motility decreased significantly in fish fed diets with genistein (table 4).

When egg characteristics were compared, the only statistical differences were observed between % of residual gonads after stripping in fish fed with 500 ppm and 1000 ppm genistein (table 5).

Fecundity and fertilisation

Fecundity and fertilisation rates in females from each group are presented in table 5. Evolution of spawning is presented in figure 5. At the first spawning date (17/12/97), only 10.5% of the fish spawned in the group fed 500 ppm genistein against 21.05 % in the group

fed the control diet and 23.5% in the group fed 1000 ppm genistein (Figure 5). Moreover, at this date, only 50% of the fish fed 500 ppm genistein yield viable eggs whereas 100% of fish of the other groups gave viable eggs. Throughout the spawning period, the percentage of spawning females in the 500 ppm fed group was always lower than in the other two groups. Over the total period of spawning, 100 % of females of the control and the 1000 ppm fed group ovulated against 79 % of the females from the 500 ppm fed group. Indeed, six females out of 19 did not ovulate and among them 3 were blocked at a previtellogenic stage and 3 were only delayed and would have probably spawned later on, after the experiment. There was no difference in fecundities. Moreover, although there was no difference in egg diameter between groups, the highest variability was observed of the 500 ppm fed group (variation coefficient were 4.23, 7.51 and 5.60 for diet with 0, 500 and 1000 ppm respectively). Fecundity was generally lower in 500 ppm fed fish. At the eyed stage, the different batches coming from the females fed the same diet were pooled and those of the worst spawns were eliminated. At that time the results obtained on embryo survival were not significantly different.

DISCUSSION

Diets

The choice of the dietary genistein levels was made in accordance with previous data showing that some soy products can contain up to 6000 ppm genistein or 8000 ppm of total phytoestrogens when daidzein was also considered (Mambrini *et al.*, 1999).

250 g of highly pure genistein were used in this experiment. Genistein (genistein) was incorporated as such in the diet whereas naturally it is present in plant by-products as glycoside, acetyl or malonyl derivatives (Anderson and Wolf, 1995). Indeed, we have been able to detect genistein in the plasma of fish fed diets containing soy protein concentrates at

317 levels ranging from 20 to 250 ng/ml using the technique reported in Bennetau - Pelissero *et*
318 *al.*, (2000). Under such circumstances, since gut microflora in fish is considered as inefficient
319 (Lesel, 1987), some hydrolysis must occur in the gut and probably in the stomach under the
320 action of digestive enzymes and low pH. This suggests that genistein is naturally
321 deglycosylated and is absorbed into the blood stream. However the bioavailability of
322 genistein with a diet enriched with either genistein or genistin (its glycoside derivative)
323 remains to be investigated.
324
325 ***Growth***
326 In this study, there was no effect of pure genistein on fish growth. In mammals, Stob, (1983)
327 reported that phytoestrogens can alter appetite via an interaction at the level of central
328 nervous system and thus can reduce daily weight increase. Our data suggest that,
329 phytoestrogens or at least genistein even present at high levels in food but were not the
330 compounds responsible for the alteration of growth, contrary to earlier observations of
331 Mambrini *et al.*, (1999) who found that high levels of dietary soy protein concentrates, rich in
332 phytoestrogens led to decreased growth of rainbow trout.
333
334 ***Cholesterol***
335 Plasma cholesterol levels in vitellogenic females and in males undergoing spermiogenesis
336 were not affected by dietary treatments. These results are in contrast to those from studies in
337 rats (Dodge *et al.*, 1996 ; Kirk *et al.*, 1998) showing that soy-protein based diet or
338 phytoestrogens can lower cholesterol blood concentrations. In rainbow trout,, Kaushik *et al.,*
339 (1995) also showed that replacement of fish meals by soy by-products had a
340 hypocholesterolemic effect. Either genistein is not the compound which, in soy, provokes
341 cholesterol lowering in the rainbow trout, or that the lowering of cholesterol was due to the

342 global decrease of cholesterol in the diet when fish meal (rich in cholesterol) was replaced by
343 soybean protein concentrates (low in cholesterol).
344
345 ***Endocrine parameters***
346 The group fed the fish-meal based diet exhibited normal steroid profile when compared to
347 data obtained on fish when soy was only a minor component of fish diets (Hoar *et al.*, 1983)
348 and classic gonadotrophin profiles (Breton *et al*., 1998).
349 The interpretation of the effect of genistein must be done remaining that it is both an
350 *in vivo* agonist and an antagonist of E_2 (Adlercreutz, 1990). *In vitro,* this dual effect seems to
351 be affected by the ratio between E_2 and genistein at the target organ (Adlercreutz *et al.*, 1995).
352 A genistein/E_2 ratio between 10 to 100 would lead to a competition for the estradiol receptor
353 (ER) which fails to induce gene transcription ; an anti-estrogenic effect is then observed
354 (Verdeal *et al.*, 1980). Below, the estrogenic effect of E_2 would mask genistein effect and
355 above genistein exerts an overall estrogenic effect. This can be explained, at least partly, by
356 the difference in affinity between E_2 and genistein for the ER. It was recently shown *in vitro*,
357 that genistein affinity is 50 times less that of E_2 for rainbow trout ER (Latonnelle *et al.*, 2000).
358 Other properties essentially demonstrated in mammals such as inhibition of
359 prostaglandin H synthase (Degen, 1990), of protein tyrosine kinase (PTK) and of protein
360 kinase C (PKC) (Osada *et al.*, 1988), differential binding to ERα and ERβ (Kuiper *et al.*,
361 1998 ; Miodini *et al.*, 1999) or SBP binding (Bennetau - Pelissero *et al.*, 1998) may play a
362 part *in vivo* in the endocrine disruption observed here.
363
364 *Endocrine and VTG profiles in females*
365 At the beginning of the study (T1 to T2), when females had low levels of circulating E_2,
366 genistein had an overall estrogenic effect shown by increased plasma VTG levels.

Between previtellogenic to vitellogenic stages (T2 and T3), E_2 levels rose and in the 500 ppm treated fish, genistein has no estrogenic effect. Since in this group, fish were found to be blocked in previtellogenesis, it is likely that it is then that genistein exerted its pre-eminent negative effect on gametogenesis. Maestro *et al.*, (1997) shown that both insulin and IGF stimulate PTK activity in early vitellogenic carp oocytes. Because genistein can act as a PTK inhibitor it could perturb this process and at least delay vitellogenesis and early yolk accumulation. Although, it remains to be seen whether the phenomenon observed in carp occurs in trout, this could explain why some fish (3 out of 19) did not undergo yolk accumulation in trout fed 500 ppm genistein.

During vitellogenesis (T3 to T4), plasma E_2 concentration is such that it probably overcame the effect of genistein.

Between T4 and T5, when E_2 levels returned to low values, genistein exerted its estrogenic activity as revealed by high VTG levels. The presence of high amounts of ER would then have led to a large estrogenic effect.

Data obtained in females for 11KT, and probably T levels at spawning can also be interpreted as reflecting a delayed steroidogenesis and consequently VTG synthesis in genistein-fed fish. These results can be compared with those obtained in sea bass (Kah *et al.*, 1994), where a delay of peak appearance of T was observed when fish were fed diet containing 23.22 g/100g of soy compared to a diet containing 13.93 g/100g of soy. It must be noted that the levels of steroid measured using the 11KT antiserum may reflect the levels of other androgens since the 11KT antibody was shown to cross-react at 6.8% with T, at 6.3% with 11β-hydroxytestosterone and at 3.5% with 5α-DHT.

Genistein effect on FSH levels at T4 and T5 in females may be explained as an estrogenic effect. Indeed, genistein as estrogen-like compound can exert an estrogenic negative feed-back on FSH release (Saligaut *et al*., 1998). As regards LH, there are at least

three possible scenarios. First, it can be considered that genistein, acting as estradiol prevents LH release via a dopamine interaction (Linard *et al.*, 1995). Secondly, according to Melamed *et al.*, (1998) PKC is involved in GnRH release and consequently in LH secretion. Genistein could then have impaired GnRH release and consequently LH release at spawning, through its PKC inhibitory effect. Thirdly, GABA induces GnRHII in gold fish brain through GABAA receptors (Trudeau *et al.*, 1993) and genistein was recently shown to be a specific inhibitor of GABAA receptors in rats (Huang *et al.*, 1999). There are thus many ways by which genistein could inhibit FSH and LH release. The complex interactions may explain why the reduction is not clearly dose-dependant. The results obtained in this study may be correlated to previous work by Kah *et al.*, (1994) showing that a diet containing 23.22 g/100g of soybean meal reduces pituitary GnRH when compared to a diet containing 13.93 g/100g. The authors attributed this effect to the ratio protein/carbohydrate. However, genistein might have been present in these diets and may have been the compound acting on this mechanism.

Because LH was significantly reduced in plasma of fish fed diets containing genistein, lower plasma levels of $17\alpha,20\beta(OH)_2$-P, maturation inducing steroid, synthesised under LH stimulation and allowing egg laying and sperm release (Zohar *et al.*, 1986), were expected.

Endocrine and VTG profiles in males

In males, at the beginning of the study, E_2 levels were very low and genistein acted as an estrogen, with a prolonged VTG synthesis. However, the low level of VTG synthesis in males was probably due to low levels of hepatic ER. Indeed, *in vitro*, phytoestrogens, including genistein, were less potent in males than in females to induce expression of both rtER mRNA and rtVTG mRNA (Bennetau - Pelissero *et al.*, 1998). In the latter study, females already had levels of rtER mRNA of 14 pg/ng of total mRNA whereas in males it was less than 1 pg/ng of total mRNA. Only concentrations of 10µM of genistein induced rtER and

417 rtVTG mRNA synthesis in males in a 24 hours exposure. It is likely that the circulating levels
418 of genistein were lower than these concentrations ; thus the induction of both ER and VTG
419 synthesis in male was low.

420 The present data suggest that genistein led to accelerated spermatogenesis. Under such
421 circumstances, a degradation of sperm motility as well as a decreased in sperm concentration
422 can be expected at spawning (Lahnsteiner *et al.*, 1998). Further, LH and $17\alpha20\beta(OH)_2P$ were
423 demonstrated to induce testicular hydration in European Sea bass (Mylonas *et al.*, 1997).
424 Then, if spermatogenesis was accelerated under genistein, testis were ready earlier in fish
425 under genistein and their time of exposure to both LH and $17\alpha20\beta(OH)_2P$ could be
426 responsible for the decrease in sperm count and increase in sperm volume. This explanation
427 may not be the only one. At full spermatogenesis (T4) the E_2 profile exhibits an increase in
428 control fish, but not in genistein fed fish. This rise is commonly interpreted in males as an
429 aromatisation of androgens into estrogens at the hypothalamic and pituitary level, inducing
430 LH release and maturation (Fostier *et al.*, 1979). However, this peak seems high and may not
431 only be due to this aromatisation process. Because, fish from all groups reached spermiation
432 and the proportion of fish ready to reproduce was higher on the 29/10/97 in treated groups, it
433 can be supposed that this E_2 rise did occur but was not seen under the sampling frequency
434 used. The VTG rise observed at T4 in all treated fish agrees with this interpretation. The
435 highly significant rise in plasma E_2 in control males at T4 is followed by a decrease at T5.
436 This decrease can be due both to a decreased aromatase activity in the brain or to a
437 disappearance of a common precursor of T and E_2 since T at T5 is also reduced.

438 The acceleration of spermatogenesis, supported by gonadal examination at T2, by E_2
439 and VTG profiles as well as by sperm characteristics, can be due, under genistein exposure, to
440 an estrogenic effect of genistein because endogenous estradiol was very low. Indeed, in
441 amphibians, one of the consequences of a slight estrogen exposure was acceleration of

spermatogenesis (Cobellis *et al.*, 1999). The estrogenic effect of genistein could also result in a similar effect in trout, as suggested by the data of Thomas (2000). The differences observed in T and 11KT plasma levels (figure2c and 2d) at spawning also suggest an acceleration of spermatogenesis. At spawning, the effect of genistein on LH release as proposed for females is also possible in males. The inhibition of LH release, although not significant, can be correlated with the significantly decreased plasma $17\alpha,20\beta(OH)_2$-P. The lack of significance might be due to the low number of fish analysed for LH measurements (10 males and 10 females only).

In both sexes, plasma testosterone levels were significantly decreased in genistein-fed fish although spermatogenesis was accelerated in males. Indeed, if spermatogenesis was accelerated with genistein exposure, an increase in T levels would have been expected. However, plasma T levels and those of other steroids reflect both secretion by the gonad and elimination by specific hepatic enzymes. Among them, T6β-hydroxylase known to play a major role, the activity of which has been found to increase when fish were fed a soy enriched diet (Cravedi *et al.*, 1997). When T levels rose the effect of genistein on T lowering was no more significant. This can possibly be due to enzyme substrate saturation. Decreased plasma T levels by estrogenic endocrine disruptors has been reported in carp captured in polluted rivers (Folmar *et al.*, 1996) and in alligators submitted to estrogenic pollutants (Guillette *et al.*, 1999), although the mechanisms involved are not clear.

Reproductive efficiency

Assessed both in terms of gamete quality and fecundity, the 500 ppm treated group presented the poorest characteristics both in males and females. Overall, the effect of genistein can be summarised as that reproduction was delayed in females and accelerated in males. It is possible to speculate on the stage of oogenesis when the effect seems to be the strongest. In

467 the 1000 ppm treated fish, there is a decrease in GTHs and 17α20β$(OH)_2$P at the end of
468 gametogenesis ; however this does not seem to affect spawning adversely. In 500 ppm treated
469 fish, although plasma LH and 17α20β$(OH)_2$P levels were decreased the greatest effect seems
470 to be an impairment in the transition of the gonad from a previtellogenic stage to
471 vitellogenesis. This is supported since among the 19 females of this group 3 did not start
472 vitellogenesis, 3 others started vitellogenesis but probably later and did not spawn by the end
473 of the trial. Two females among those which spawned gave poor quality laying with very low
474 survival rates at the eyed stage (about 0%). In addition, the heavier residual gonad was found
475 in this group. This probably reflects that, in the spawning females, the eggs were not all
476 synchronised by the time of spawning. In males, the acceleration of spermatogenesis seems to
477 occur at early spermatogenesis as revealed by histology. Although the frequency analysis did
478 not reveal significant differences, similar results obtained with soy based diets in male
479 rainbow trout (unpublished results) also suggest the same effect. The slight GTHs and
480 17α20β$(OH)_2$P impairment could also reduce male reproductive performance in the 500 ppm
481 treated group.
482
483 This study showed that genistein, a natural endocrine disruptor of dietary origin, has some
484 effects on the reproductive performances of the rainbow trout at the doses tested. No clear
485 dose dependant response were recorded, with doses of 500 ppm having more deleterious
486 effects on reproductive efficiency than doses of 1000 ppm. Data show that the effect of
487 genistein on the gonadal development depends greatly upon the stage and the endogenous
488 estrogen secretion.
489 In fish farms trout reared until 250g mean weight are likely not to be influenced by
490 soy isoflavone in their diet (no growth disruption in this trial). In triploid fish the effect of

491 isoflavones remain to be tested but since reproduction is not completed it can be expected that
492 soy based diet would not have major effects.

493 Finally, even if genistein has only minor effects on the trout reproductive
494 performances phytoestrogens interactions with steroids have to be considered in spawners
495 especially when reproductive physiology criteria are analysed in endocrine approaches.

496

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504

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Figure and Table Captions

Figure 1. Chronology of sample times

Figure 2. Steroid profiles in male rainbow trout submitted to KB1 : control fish-meal based diet, KB2 :diet with 500 ppm genistein, KB3 : diet with 1000 ppm genistein. Sample times are presented in figure 1. Values are means of 20 measurements; bars are sem, * indicates a significant difference at α=0.05.

Figure 3. Steroid profiles in male rainbow trout submitted to KB1 : control fish-meal based diet, KB2 :diet with 500 ppm genistein, KB3 : diet with 1000 ppm genistein. Sample times are presented in figure 1. Values are means of 20 measurements; bars are sem, * indicates a significant difference at α=0.05.

Figure 4. Frequency distribution of testis developmental stages in males submitted to KB1 : control fish-meal based diet, KB2 : with 500 ppm genistein, KB3 : with 1000 ppm genistein. The significance of the stages is given in table 3. For each diet 20 fish were considered at each sample time.

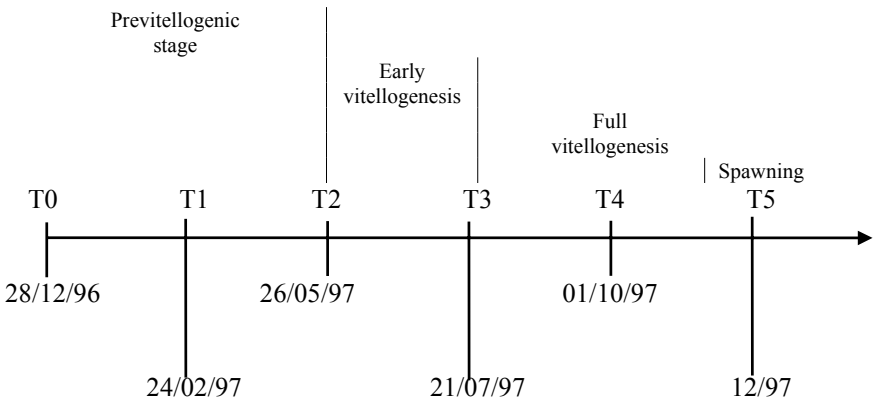
Figure 5. Performance of spawning of female rainbow trout submitted to KB1 : control fish meal based diet, KB2 : with 500 ppm genistein and KB3 : with 1000 ppm genistein. Value sharing a common letter are significantly different; at α=0.1.

Table 3. KB1 is a control fish-meal based diet, KB2 is a diet with 500 ppm genistein, KB3 is a diet with 1000 ppm genistein. For GTHs values are means ± sem of 10 measurements, for 17α,20β(OH)$_2$-P values are means ± of 19 measurements.

Table 4. KB1 is a control fish-meal based diet, KB2 is a diet with 500 ppm genistein, KB3 is a diet with 1000 ppm genistein. Values are means of 19 males ± sem.

Table 5. KB1 is a control fish-meal based diet, KB2 is a diet with 500 ppm genistein, KB3 is a diet with 1000 ppm genistein. Values are means of 19 males ± sem.

Figure 1.


Previtellogenic stage
Early vitellogenesis
Full vitellogenesis
Spawning
T0
T1
T2
T3
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T5
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21/07/97
01/10/97
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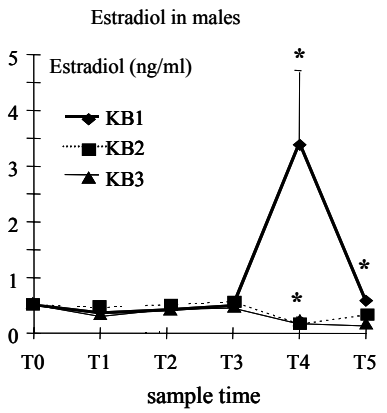
Figure 2

2a



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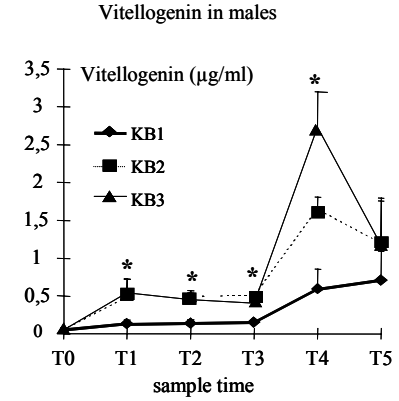

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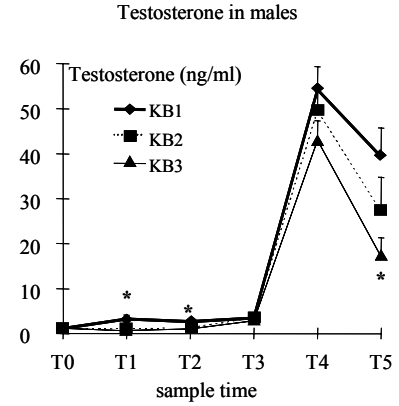

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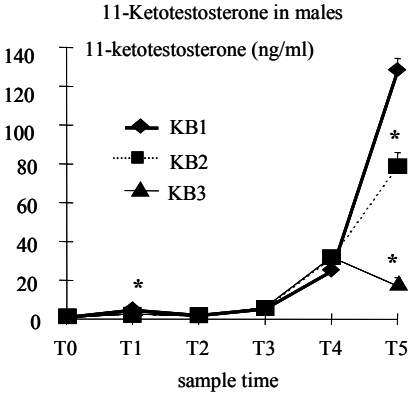
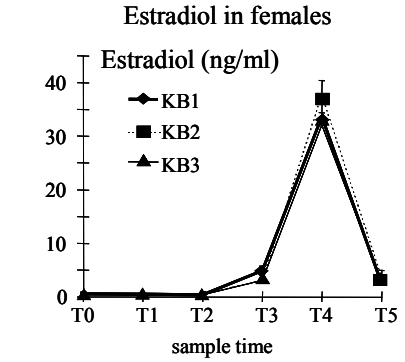


812 **Figure 3**

813 **3a**



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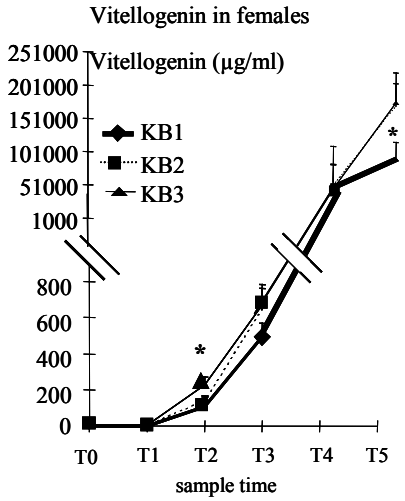

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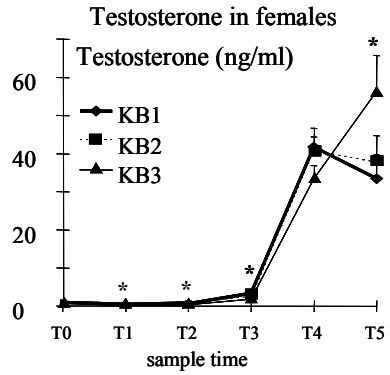

826 **3d**

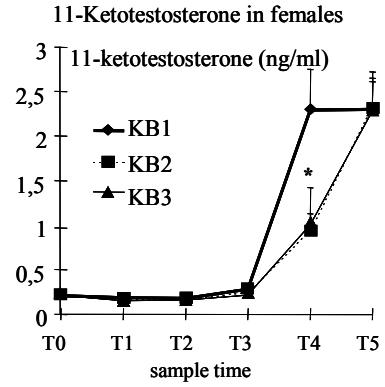

Figure 4

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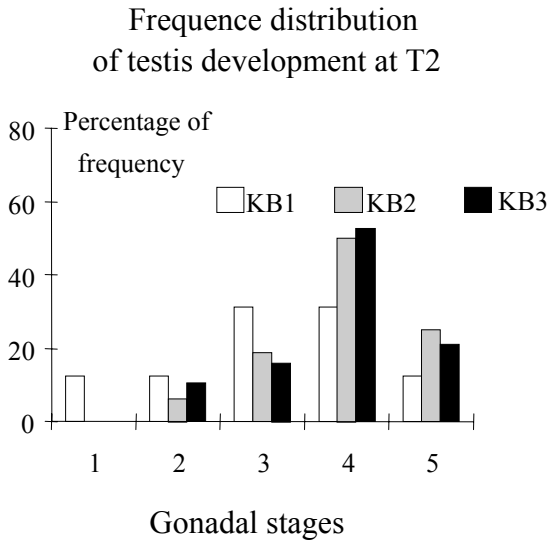

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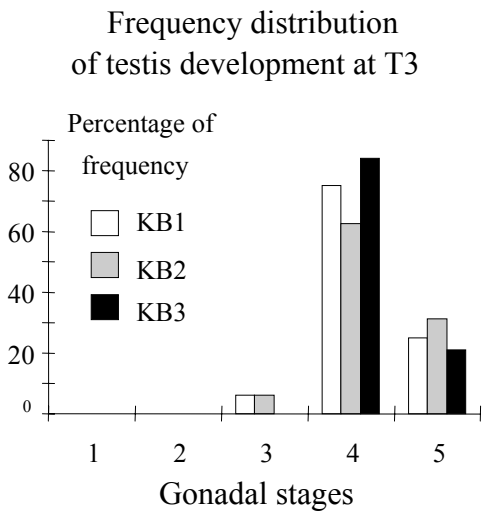

849 **Figure 5**

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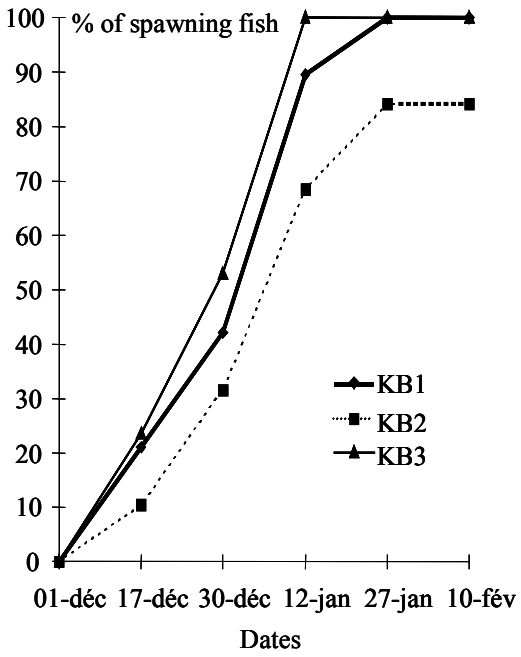
851 Evolution of spawning during the spawning period



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