Effects of α-naphthoflavone on body growth and gonad development in chickens (*Gallus domesticus*)

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ABSTRACT: The aim of this study was to investigate the effects of prolonged oral administration of α-naphthoflavone on somatic and germinal development in male and female chickens (*Gallus domesticus)*. No significant differences were observed at any tested age (6, 8, 10 and 12 wks) between control and treated groups of either sex in total body weight and in the weights of kidney, liver, gizzard, heart, abdominal fat, breast and ovary tissues ($P > 0.05$). Mean testicular weights were similar between groups at 6, 8 and 10 wks but at 12 wks males supplemented with α-naphthoflavone had significantly heavier testicle weights ($P < 0.01$) compared to 2 control groups. Histological examination of the seminiferous tubules (ST) revealed a more advanced stage of maturation in the testes of α-naphthoflavone-treated males, and the presence of testicular spermatozoa was observable as early as at 12 weeks of age. The testes of males from the two control groups were still at an impuberal stage at this age.

Keywords: *CYP 19* inhibitor; body growth; testes; broiler breeders; poultry

Flavonoids are a group of phytochemicals exhibiting a wide range of biological activities including antioxidant properties and ability to change several enzymes and cell receptors. α-naphthoflavone (ANF) is a flavonoid that was reported to inhibit the expression of *CYP11A* gene (Hodek *et al*., 2002) which encodes for an enzyme, P450scc, controlling the cleavage of a mitochondrial side-chain of cholesterol by multiple monooxygenations. P450scc is localized in a variety of tissues including the gonads, adrenal glands and placenta (Hinshelwood, 1999). *CYP* genes include several gene-encoding enzymes of the P450 family. They participate in steroid hormone production through a reaction cascade leading to the production of dehydroepiandrostenedione (DHEA) and androstenedione, the latter triggering the production of androgens (e.g. testosterone) and oestrogens (e.g. oestrone). The biosynthesis of steroid hormones starts in the adrenal glands, gonads and several other somatic tissues with the conversion of cholesterol into pregnenolone under the influence of cytochrome P450 *CYP11A1* (Lambeth, 1986). Oestrone, the first member of female sex hormones, is formed by a unique cytochrome P450 *CYP19* (aromatase), which builds a phenolic Ring A of all oestrogens. This enzyme is present in the ovarian tissue of both mammals and birds. The structure of this essential cytochrome is remarkably conserved in all species, and 92% homology has been observed between chicken and human *CYP19*. It is hypothesized that changes in *CYP19* activity significantly affect the oestrone/testosterone ratio and consequently induce phenotypic changes in the sex of poultry species. Several reports have indicated that the inhibition of *CYP19* activity or of its expression causes serious hormonal status changes. For example, in female chickens complete ovariectomy results in phenotypic sex-reversal accompanied by a significant increase in plasma steroids (Wallenburg,

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1982). Similarly, *in ovo* treatment of female embryos with Fadrozole, a non-steroidal aromatase inhibitor, resulted in the development of male gonads (Wartenberg *et al*., 1992; Dewill *et al*., 1998; Vaillant *et al*., 2001). However, in the majority of cases this transformation was reversible in mature birds (Burke and Henry, 1999), an indication that a single treatment of embryos with aromatase inhibitor cannot durably convert genetic females into sexually mature neo-males. Therefore there arises a question whether repeated treatment performed with aromatase inhibitors can induce permanent sex reversal in genetically female chickens or not. Since *CYP19* has been detected in males, namely in the testes, administration of *CYP19* inhibitors might be expected to change the hormone status and perhaps to induce phenotypic changes in male chickens by enhancing testosterone production through the conversion of oestrone into androstenedione. Studies in male rats treated with *CYP19* inhibitor revealed a significant decrease in plasma oestradiol (–18%) and a concomitant increase in plasma testosterone (Turner *et al*., 2000). According to our knowledge, no studies of this type have been carried out in male chickens yet. However, it can be expected that changes in *CYP19* in this species would yield similar results to mammals.

Based on previous observations that α-naphthoflavone inhibits the *CYP11A* gene (Hodek *et al*., 2002), we carried out this experiment to study the consequences of repeated postnatal oral administration of α-naphthoflavone for body growth and sexual development in male and female chickens.

MATERIAL AND METHODS

Birds and husbandry

A total of 90 day-old chicks (ROSS 308, Ross-Aviagen, Newbridge, Midlothian, UK) with similar body weights (28 g ± 1 g) were sexed and then divided equally (30 birds/group) into 3 groups/sex origin, referred to as Male Group – MG1, MG2, MG3 and Female Group – FG1, FG2, FG3. Apart from the first 3 weeks after hatching during which all birds were constantly lit by heaters, all groups were subjected to natural day-length (from 14L:10D to 11L:13D) until 12 wks of age (end of experiment). During the first 6 post-hatching wks, birds were fed daily a standard starter diet (23% CP, ME 3 100 cal/kg) provided *ad libitum* with the following specifications: birds in MG1 and FG1 were fed the standard diet only (negative control), birds in MG2 and FG2 were fed the standard diet 5 days/wk and the standard diet + drug vehicle (positive controls) 2 days/wk. The drug vehicle consisted of a mixture of 20% gelatine (v/V), 20% olive oil, 30% glycerol and 30% H_2O administered orally (syringe) at 1 ml/bird. Birds in MG3 and FG3 were fed the standard diet 5 days/wk and the standard diet + drug vehicle + α-naphthoflavone (60 mg/kg body weight) 2 days/wk. Diets with supplementation were provided on 2 non-consecutive days/wk. All birds were weighed individually once a week from hatching to 12 wks of age.

Histological preparation and stereological analyses

In all groups, a total of 5 birds/group were sacrificed (lethal dose of pentobarbital: 1 ml/kg body weight) at 2, 6, 8, 10 and 12 wks of age and their liver, kidneys, gizzards, hearts, testes, ovaries, abdominal fat and breasts were weighed (nearest mg) for inter-group comparisons.

Tissue samples from the left testis of birds sacrificed at 2, 6 and 12 wks of age were placed in Bouin's fixative for 24 hours (Bancroft and Stevens, 1982), dehydrated and then embedded in Paraplast (Sigma, St. Louis, Missouri). Four histological sections (7 μm thick) were obtained with a Leica 2000 microtome (Leica, Vienna, Austria) and then stained with Periodic Acid-Schiff (McManus, 1946) for further examination of the seminiferous tubules (ST) and of spermatogenesis.

The mean surfaces of ST present in each field were examined at × 200 magnification with an Olympus microscope equipped with an Image Processing and Analysis System (Laboratory Imaging LUCIA, version 4,60, Czech Republic). We also determined the total length (L) of ST in each male from the percentage of testicular tissue containing ST. The lengths of seminiferous tubules were determined from 10 orthogonal sections of ST/testis (round appearance of the tubules) as previously reported by Brillard (1986). Briefly, the percentages of tissue sections occupied by ST were determined with a 25 point Hennig grid from 32 randomly chosen fields/testis (× 200). ST diameters were measured with a precalibrated ocular scale from 10 randomly chosen cross sections per testis (× 200). Individual values for each parameter were expressed as the combination of the right and left testes for each male.

Hormone analyses

Blood samples from 5 individuals in each male group were collected at 2, 4, 6, 8, 10 and 12 wks of age to assess plasma testosterone levels (radioimmunoassays performed with Immunotech kit, cat. No. 1119).

Statistical analyses

Statistical comparisons of body and organ weights including seminiferous tubule parameters between groups at a given age were performed by analysis of variance and *t*-tests (Microsoft Excel 2000).

All experiments were performed in accordance with CZ legal requirements (Acts 246/1992, 162/1993 and 193/1994).

RESULTS

With the exception of testicle weights, the comparison of body and tissue weights (liver, kidney, gizzard, heart, testes, ovary, abdominal fat and muscle breast) between groups revealed no significant differences within each sex at any tested age (2, 6 and 12 wks of age; Figures 1, 2 and 3; results not shown for liver, kidney, gizzard, heart abdominal fat and muscle breast weights). In addition, the gross appearance of the ovary in hens was similar in the 3 female groups at each age.

Testicle weights in MG1 and MG2 were similar at every tested age ($P > 0.05$), and plasma levels of testosterone were also similar in these two groups. By contrast, at 12 wks of age the testes from MG3 were significantly heavier compared to MG1 or MG2 (MG3 8.2 ± 0.8 g, 2.2 ± 0.6 g and 2.3 g ± 0.05 g in MG3, MG1 and MG2, respectively; $P < 0.01$). However, this was not accompanied by any significant differences in plasma testosterone levels between treatment groups at any tested age ($P > 0.05$; Figure 4). On the basis of the above observations, we decided to establish comparisons of the testicular parameters only between MG1 and MG3.

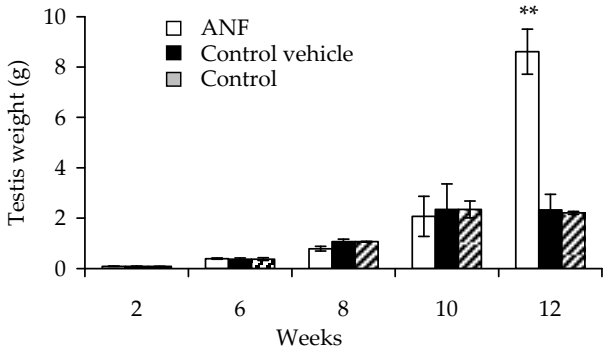
The dimensions of the seminiferous tubules are reported in Table 1. Photographs of testicular tissue from MG1 and MG3 males at 2, 6 and 12 wks of age are shown in Figure 5.

Significant differences were observed in the development of seminiferous tubules between MG1 and MG3 at 2, 6 and 12 wks of age, revealing that the oral administration of ANF between 1 and 6 wks of age was followed by earlier maturation of the tes-

Table 1. Percentage of testicular tissue occupied by seminiferous tubules (%ST) and mean length of seminiferous tubules (ST Length) in chicken males with and without α-naphthoflavone supplementation

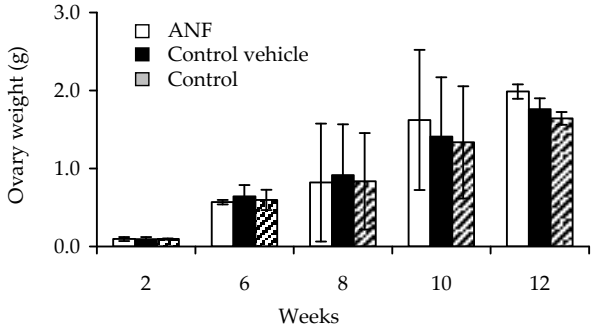
Age (weeks)	% ST		ST Length (m)	
	ANF	Control	ANF	Control
2	48.5	45.7^{ns}	0.76	1.18
6	79.4	64.0**	5.3	6.37
12	86.3	74.0**^{B}	30.3	25.4**

**$P < 0.01$ at a given age



**α-naphthoflavone treated group significantly different from the other groups ($P < 0.01$)

Figure 1. Mean testicle weights [(right + left testis)/2 in g] in male chickens with and without α-naphthoflavone supplementation



$P > 0.05$ at all ages tested

Figure 2. Ovary weights in female chickens with and without α-naphthoflavone supplementation

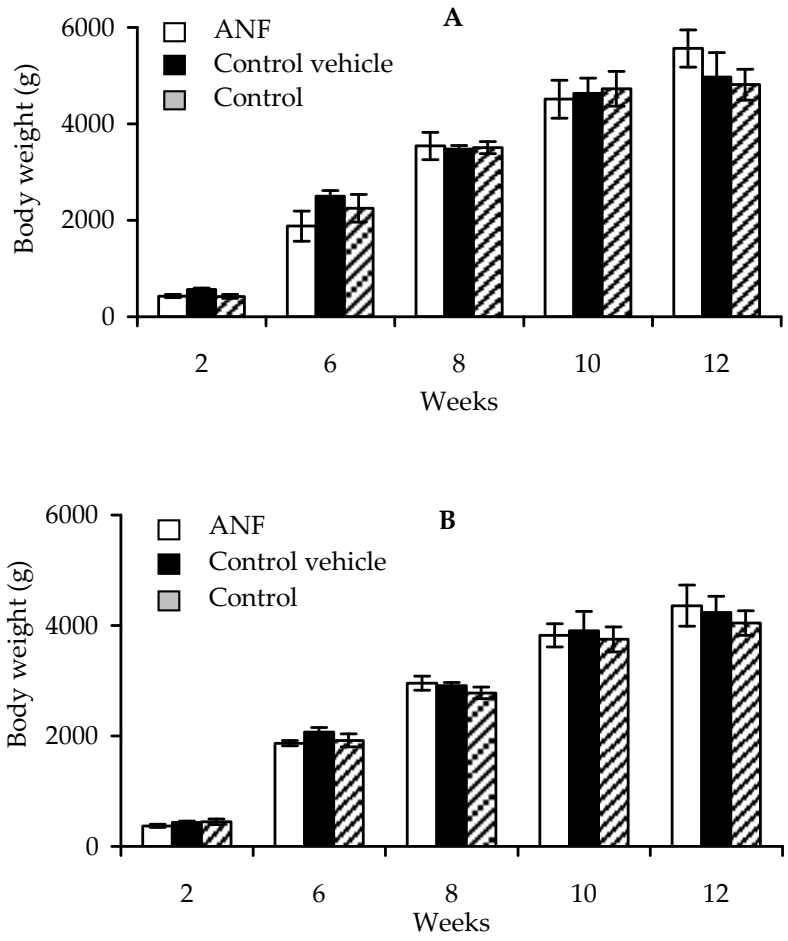


Figure 3. Body weights of male (A) and female (B) chickens with and without α-naphthoflavone supplementation

$P > 0.05$ at all ages tested

tes in MG3 compared to MG1 (Figure 5). Thus, the surface of seminiferous tubule epithelium/field was significantly larger in testes from MG3 compared to MG1 males at 12 weeks ($P < 0.01$; Figure 6), and ST dimensions (diameter, TL) were therefore increased in the testes from MG3 compared to MG1 males at 12 weeks ($P < 0.01$).

Earlier development of the testes in MG3 males was accompanied at 12 wks of age by the presence of luminal spermatozoa in the testes of 4/5 of males sacrificed at this age. By contrast, spermatogenesis in the testes from MG1 males at this age was limited to the presence of type-I spermatocytes in a diplotene stage. From the behavioural aspect, it

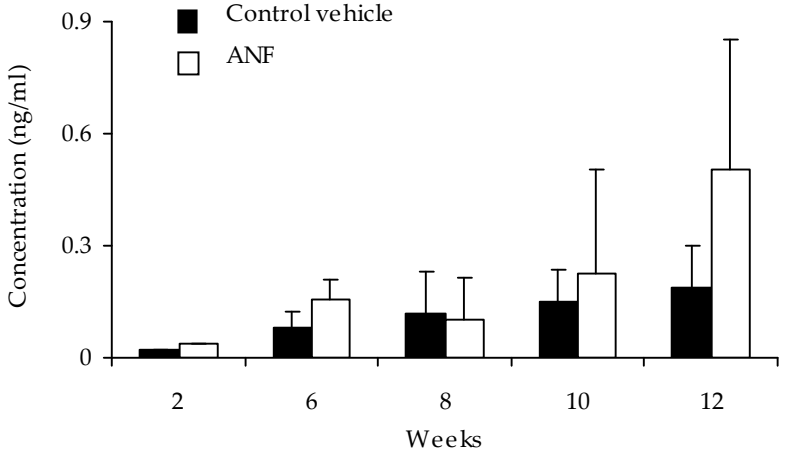


Figure 4. Plasma testosterone levels in male chickens with and without α-naphthoflavone supplementation. Plasma testosterone concentration (means ± SEM) was examined at 2, 6, 8, 10 and 12 wks of age

$P > 0.05$ at all ages tested

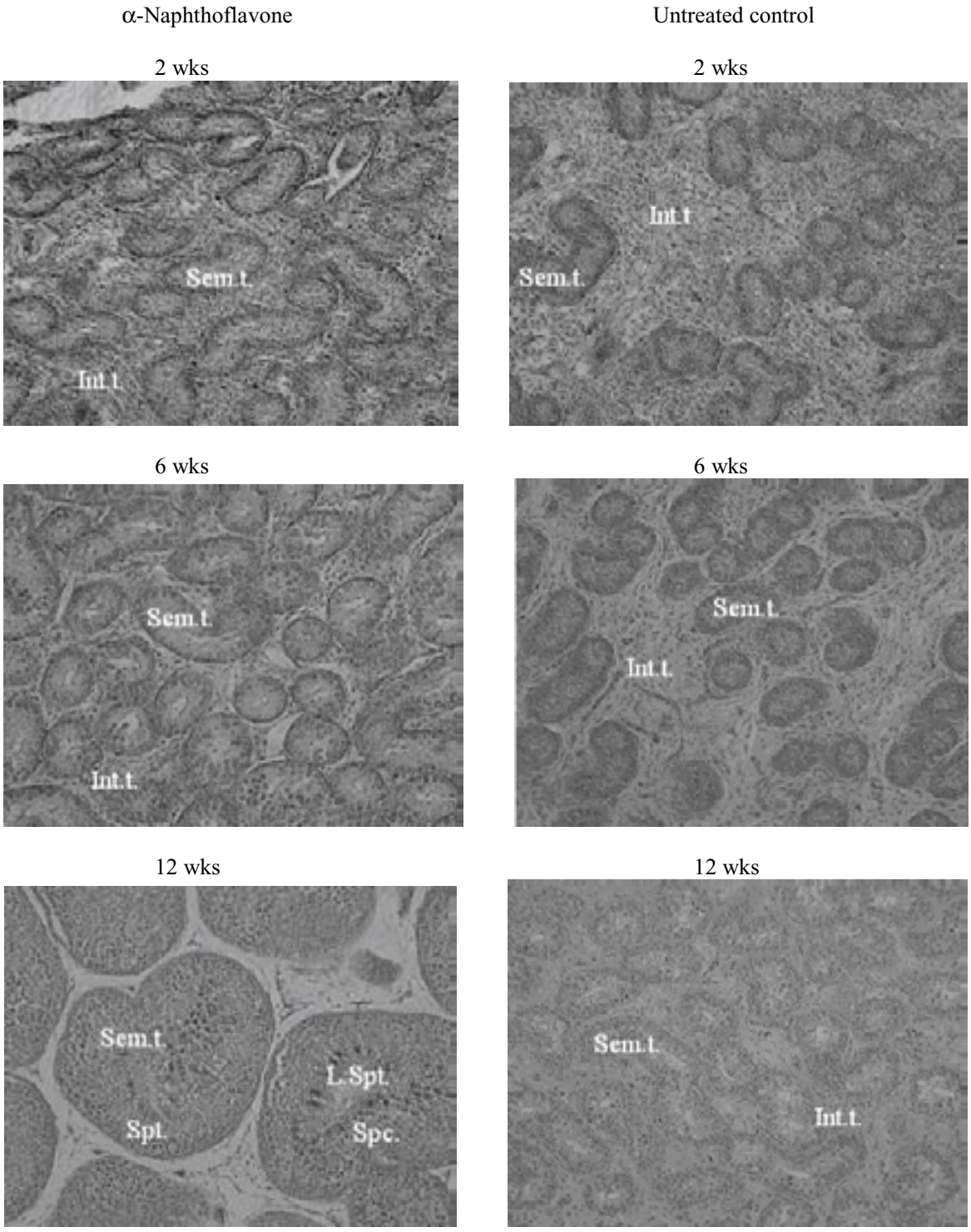


Figure 5. Testicular tissue in chickens supplemented with α-naphthoflavone at 2, 6 and 12 weeks of age (PAS stain, × 200)

Sem. t. = seminiferous tubules; Int. t. = interstitial tissue; Spc. = type-I spermatocyte; Spt. = round spermatids; L.Spt. = elongated spermatids
Note the bundles of elongated spermatids in the luminal portion of the tubules in an α-naphthoflavone treated male at 12 wks of age

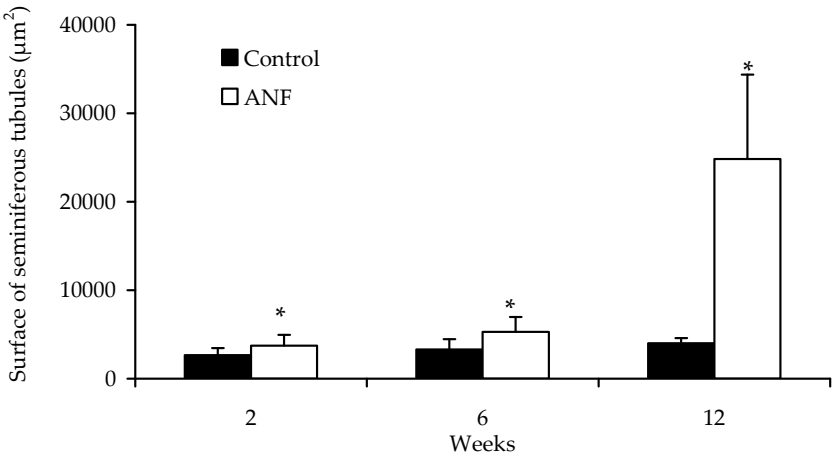


Figure 6. Comparison of seminiferous tubule surface in tissue sections from testicles of male chickens with and without α-naphthoflavone supplementation

*α-naphthoflavone treated group significantly different from the other groups ($P < 0.01$)

was noticeable that only males from MG3 started to crow at this age.

DISCUSSION

With the exception of the testes of males supplemented with α-naphthoflavone, the oral administration of this flavonoid for 6 wks after hatching had no apparent effect on body or tissue growth until 12 wks of age, irrespective of the genetic sex. Testicular development in chickens is at first controlled by the photoperiod but factors such as genetic origin or feed allocation may also exert an influence (see Brillard, 2003 for review). Puberty, revealed by the presence of luminal spermatozoa in the seminiferous epithelium, can be observed as early as at 12 wks of age in males first subjected to a long photoperiod (e.g. from 16L:8D, de Reviers, 1971). By contrast, puberty is delayed by 4–6 wks in chicken males subjected to decreasing photoperiods (e.g. from 16L:8D to 8L:16D) (de Reviers, 1996). In the present study, neither the genetic origin (males from a fast growth rate broiler-type) nor feed allocation (*ad libitum*) can be considered as having exerted a retarding effect on gonad development. In addition, the photoperiod environment of these males was probably non-stimulatory according to the absence of spermiogenesis in the testes of males from the two control groups. It can therefore be postulated that the occurrence of qualitatively normal figures of spermiogenesis in 4 out of the 5 males treated with α-naphthoflavone was a direct consequence of the treatment rather than originating from inter-individual variability of these males in response to the photoperiod.

Previous studies demonstrated that sperm production was highly correlated with testicle development (de Reviers, 1996). As a consequence, males with large testes generally produce more spermatozoa than males with limited development of the testes. In chickens, the development of seminiferous tubules is mainly under the influence of gonadotropins (LH and FSH) while Leydig cells are responsible for the production of male steroids (Knobil and Neill, 1998). However, in the present study, plasma testosterone levels were not significantly different between males treated with α-naphthoflavone and control males. This may initially seem to contradict previous observations. In our opinion and in view of the large differences observed at the testicular level at 12 wks of age, this contradiction was more probably due to high inter-individual variability within each group and/or to the small number of individuals/treatment rather than to the absence of the effect of treatment at this stage. Testosterone and hydroxy-derivates secreted by Leidig cells play an important role in the occurrence of secondary sex characteristics in avian species (Kuhn, 2002). In this study, the fact that males treated with α-naphthoflavone started to crow at 12 weeks can be considered as a consequence of the rapid testicle development in this group which, despite the absence of significant differences from other groups, was accompanied by earlier development of steroid-dependent behaviour in these males.

Finally, our results suggest possible involvement of P450 enzyme in gonad stimulation in chicken males. However, due to the absence of significant differences in testosterone concentrations between the treatment groups, we cannot draw any conclusion about the existence of the effect of α-naphtho-

flavone as an inhibitor of P450 enzyme to convert testosterone into oestradiol. Further studies with larger numbers of birds per treatment are therefore needed to confirm this hypothesis.

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ABSTRAKT

Vliv α-naftoflavonu na tělesný růst a na vývoj gonád u kuřat (*Gallus domesticus*)

Cílem této studie bylo ověřit vliv opakovaně perorálně podaného α-naftoflavonu na tělesný a pohlavní vývoj samců a samic kura domácího (*Gallus domesticus*). Mezi aplikovanými skupinami a kontrolními skupinami nebyly v průběhu pokusu ve věku 6, 8, 10 a 12 týdnů zjištěny statisticky významné rozdíly ($P > 0,05$), a to nejen v tělesné hmotnosti jedinců obou pohlaví, ale i v hmotnostech ledvin, jater, žaludku, srdce, abdominálního tuku, prsního

svalu a vaječníku. Naproti tomu byly zjištěny, v porovnání s kontrolními skupinami, statisticky významně vyšší ($P < 0,01$) průměrné hodnoty hmotností varlat u α-naftoflavonem ošetřených skupin jedinců od 12. týdne věku. U skupin kohoutů ošetřených α-naftoflavonem byla od 12. týdne věku rovněž histologicky potvrzena pokročilejší stadia zrání semenných kanálků (ST) a přítomnost spermií ve varleti v porovnání s kontrolními skupinami.

Klíčová slova: *CYP 19* inhibitor; tělesný růst; varlata; brojleři; drůbež

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